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ORIGINAL ARTICLE

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Surface mesoderm in *Xenopus*: a revision of the stage 10 fate map

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Abstract We have used two complementary cell labeling techniques to investigate dorsal mesoderm formation in *Xenopus laevis* and *Hymenochirus boettgeri*. Epithelial grafts from fluorescently labeled donors into unlabeled hosts demonstrate that in *Xenopus*, as previously shown for *Hymenochirus*, superficial cells of the dorsal marginal zone have the ability to invade the notochord and somite and participate in their normal morphogenesis, in a stage-specific and region-specific manner. A new method for superficial fate mapping using cell surface biotinylation confirms this result for *Hymenochirus* and demonstrates that in *Xenopus* as well, even in normal development in the absence of surgical disruption, notochord and the most posterior somitic mesoderm originate partly in the superficial epithelial layer. This finding is contrary to the widespread belief that *Xenopus* mesoderm originates solely in the deep mesenchymal layer. In *Xenopus* (but not in *Hymenochirus*), the amount of superficial contribution to mesoderm varies, such that in some spawnings it appears not to be present, while in others it is evident in all or most embryos.

Key words Biotin · Notochord · Somites · *Xenopus* · *Hymenochirus*

Introduction

It is generally accepted that the mesoderm in *Xenopus laevis* arises solely from the deep mesenchymal layer of the involuted marginal zone, while the superficial epithelial layer lining the archenteron contributes only to endoderm (Nieuwkoop and Florschütz 1950; Keller 1975, 1976). It has been further argued that *Xenopus* represents a model for all anurans (Løvtrup 1965, 1966, 1975; Nieuwkoop and Sutasurya 1976), but the accumulating evidence for diverse anuran species has re-established the older idea that the notochord and somites generally have a dual origin, incorporating cells from both the deep and superficial layers (Purcell 1992; Purcell and Keller 1993; Delarue et al. 1994, 1996). Most recently, we used fluorescently labeled tissue grafting experiments and time-lapse video micrography to demonstrate such a dual mesodermal origin for *Hymenochirus boettgeri*, a close relative of *Xenopus* (Minsuk and Keller 1996).

Even in *Xenopus*, contradictory evidence exists. Smith and Malacinski (1983) presented data indicating that superficial cells contributed to the *Xenopus* notochord. *Xenopus* is the chief amphibian developmental model system, and the presumptive dorsal mesoderm is also the classical organizer (Spemann and Mangold 1924, translated in Willier and Oppenheimer 1974), responsible for the patterning of the lateral and ventral mesoderm as well as the induction and patterning of the central nervous system. The cellular and molecular processes involved in these events are a focus of modern studies and are of relevance to axis development not just in amphibians but in all vertebrates (Graff 1997; Poznanski et al. 1997; Sasai and De Robertis 1997). It is therefore critical to identify the cell populations involved in dorsal mesoderm development and to understand their developmental histories, their morphogenetic movements, and the interactions between them.

In order to clarify the status of *Xenopus*, and in order to make possible a direct comparison with *Hymenochirus*, we have duplicated in *Xenopus* the same tissue grafting experiments earlier performed in *Hymenochirus* (Minsuk and Keller 1996). We find that labeled, grafted superficial marginal zone (SMZ) contributed to the mesoderm in a stage- and region-specific manner similar to that in *Hymenochirus*, though with more variability. In addition, we have devised an alternative method of mapping superficial cell fates using cell surface biotinylation

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to label cells, thus avoiding the mechanical disruption inherent in grafting. In *Hymenochirus*, this method confirms the consistent contribution of the SMZ to notochord and somites; in *Xenopus*, it reveals a variable SMZ contribution to notochord and to the most posterior somitic mesoderm. These results demand a revision of the established belief that all mesoderm in *Xenopus* arises in the deep layer.

Materials and methods

Handling of adults and embryos

X. laevis were kept and induced to ovulate, and their eggs fertilized in vitro and dejellied, according to standard procedures (Kay and Peng 1991). *H. boettgeri* adults and embryos were handled as previously described (Minsuk and Keller 1996). Briefly, male and female adults were both injected with 0.06 ml (500 IU/ml) human chorionic gonadotropin (Sigma) and allowed to mate naturally. Embryos were handled much like those of *Xenopus* except that 0.1% bovine serum albumin (BSA) was added to most solutions to reduce stickiness. *Hymenochirus* embryos were always dejellied, stored, and handled at room temperature.

Embryos were staged according to Nieuwkoop and Faber (1967). As previously described (Minsuk and Keller 1996), the *Xenopus* staging table is adequate for *Hymenochirus* up through early tailbud stages.

Fluorescent labeling

Xenopus embryos were labeled by injection of 70 or 10 kDa, lysine-fixable fluorescein- or rhodamine-conjugated dextran amine (Molecular Probes; Gimlich and Braun 1985). Fertilized eggs were dejellied and placed in 100% modified Barth's solution (MBS; Gurdon 1977) plus 0.1% BSA and 5% ficoll. Label was air-pressure injected into each embryo using a micropipet needle mounted on a micromanipulator, similar to the method previously described (Keller and Tibbetts 1989). After about 1 h, embryos were transferred into 33% MBS plus 0.1% BSA and 5% ficoll.

Epithelial grafts

Homotopic grafts of *Xenopus* SMZ were made from fluorescently labeled donor embryos into unlabeled host embryos of the same stage. Dorsal marginal zone (DMZ) grafts were done between stages 10 and 10.25, and lateral marginal zone (LMZ) grafts between 10+ and 10.5. When necessary, embryos were transferred into Winklbauer's dissociation medium (Winklbauer 1988), a Ca^{2+}/Mg^{2+}-free buffered saline, to facilitate the clean removal of epithelial tissue with a minimum of attached deep mesenchymal cells; otherwise surgery was performed in 33% MBS plus 0.1% BSA. An eyebrow-hair knife was used to gently tease back and remove the DMZ or LMZ epithelium from the host (Fig. 1A), and the host was then transferred immediately into Sater modified Danilchik's solution (Sater et al. 1994) plus 0.1% BSA. A similarly-sized piece of epithelium from the same position was then removed from the labeled donor embryo, taking special care to remove all adhering deep cells, and the explanted epithelium was transferred to Danilchik's solution and gently placed onto the exposed mesenchymal region of the host in its normal orientation (Fig. 1B, C). All surgery was performed on a cold plate kept at 16–18°C. The grafted embryos were then allowed to heal and gastrulate in the dark, at either room temperature or 16°C. Around the end of gastrulation (stage 13–14), embryos to be cultured for a longer time were transferred into 33% MBS plus 0.1% BSA.

Two additional types of grafts were made in a similar manner. Ventral animal cap epithelium was grafted homotopically, from labeled donors at stage 10 (or as soon as possible thereafter, as late as stage 12), into the corresponding positions in the animal caps of unlabeled hosts of the same stage (Fig. 1E). In addition, heterotopic epithelial grafts were performed from the donor DMZ into the host ventral marginal zone (VMZ), between stages 10 and 10.25 (Fig. 1D).

After subsequent fixation, embryos were sectioned either sagitally or transversely, and were scored by counting the number of labeled cells clearly identifiable in the mesoderm. In certain cases, such as where adjacent cells were labeled, a precise count was not possible; the numbers reported are low estimates and therefore conservative.

Biotinylation of the superficial layer

Hymenochirus and *Xenopus* embryos were biotinylated either at stage 9 or 10, or immediately after fertilization. *Hymenochirus* embryos that had been developing in medium containing BSA were first rinsed a few times in plain 33% MBS in order to remove as much BSA as possible, to prevent it from competing with the embryo's own surface proteins for biotinylation. Dejellied embryos were placed, with their vitelline envelopes intact, into 1 ml sulfo-NHS-LC-biotin (Pierce Chemical Co.), 5–10 mg/ml in 33% MBS (prepared immediately before use to prevent hydrolysis of the reagent) at room temperature for 30 min, with gentle agitation. Embryos were then rinsed three times with 10 mM glycine in 33% MBS to quench the reaction, and transferred back into plain 33% MBS. Embryos were allowed to develop to the desired stages, and their vitelline envelopes were removed with watchmaker's forceps prior to fixing.

Viability controls.

In the experiments described below, a minimum of 5–10 biotinylated embryos and a similar number of unlabeled embryos from each spawning were set aside and allowed to develop for about a week. Dead embryos were removed from the dishes and solutions were replaced when dirty. There was some mortality under laboratory conditions but survival rates were not detectably different in labeled embryos as compared to unlabeled embryos, in any spawning of either species. Surviving tadpoles (stage early 40's) were completely normal in their morphology and their swimming behavior. Therefore, the biotinylation procedure was not toxic and did not interfere with development, at least on a gross level, either in *Hymenochirus* or in *Xenopus*.

Fixation and histology

Unlabeled, fluorescently labeled, or biotin-labeled specimens were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), dehydrated through an ethanol series or transferred directly into 100% ethanol, cleared in Histosol, embedded in Paraplast, and sectioned at 10–20 μm. Fluorescently labeled specimens were viewed under epifluorescent optics on a Nikon inverted microscope.

Biotin visualization

The biotin-avidin interaction has usually been used to identify or isolate proteins in vitro; rarely has it been used to locate the labeled proteins in situ in whole organisms. Flaherty and Swann (1993) visualized biotinylated proteins on the surface of mouse oocytes, live or immediately after fixation. We know of no study in which the biotin signal was tracked to the interior of cells or tissues, or in which it was required to survive histological procedures, as is the case here. We therefore devised a protocol for the in situ visualization of the biotin signal in sectioned material, modeled after existing whole-mount immunohistochemistry pro-

cedures (Hemmati-Brivanlou and Harland 1989; Hemmati-Brivanlou et al. 1990) and modified for sections, with the biotin-streptavidin interaction treated as analogous to the interaction between a secondary and primary antibody.

Sections were de-waxed in xylene and rehydrated through an ethanol series to pure distilled water, then to TRIS-buffered saline (TBS: 100 mM TRIS pH 7.4, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20) for 5 min, and finally were blocked in TBS plus 1% BSA for 1 h to prevent non-specific streptavidin binding. Slides were then placed horizontally in a humid chamber, raised away from any flat surface using a plastic support. In each of the following steps, each slide was covered with 0.5 ml solution, and the lid was replaced on the chamber. After the specified immersion time, the solution was poured off of each slide, and the slide tipped on edge against a paper towel to remove the remaining liquid, prior to replacing the slide in the chamber for the next solution. Solution changes were done quickly to avoid drying out the sections.

Slides were first covered with alkaline phosphatase-conjugated streptavidin (Vector Labs) diluted 1:500 in TBS plus 1% BSA for at least 1 h. Slides were then washed with TBS three times for at least 15 min each, rinsed in alkaline phosphatase buffer (AP-buffer: same recipe as TBS except using TRIS pH 9.5, and with 5 mM levamisole added immediately before use to inhibit endogenous phosphatases), and covered with fresh AP-buffer for 5 min. The color reaction was then performed by covering the slides with AP-buffer containing, per milliliter, 4.5 µl NBT (nitro blue tetrazolium, 75 mg/ml in 70% dimethyl formamide) and 3.5 µl BCIP (5-bromo-4-chloro-3-indolyl phosphate, 50 mg/ml in 100% dimethyl formamide).

The color reaction was observed through a stereoscope and stopped when the dark blue precipitate became apparent, typically after 60–90 min, by pouring off the reaction buffer and immersing the slides in 5 mM EDTA in TBS for at least 20 min. Slides were then transferred to distilled water and dehydrated through ethanol, xylene and toluene before mounting cover slips with Pro-Texx mounting medium (American Hospital Supply Corp.). Specimens were viewed under DIC optics and digitally recorded through a Zeiss Axioplan microscope using a Sony CCD video camera.

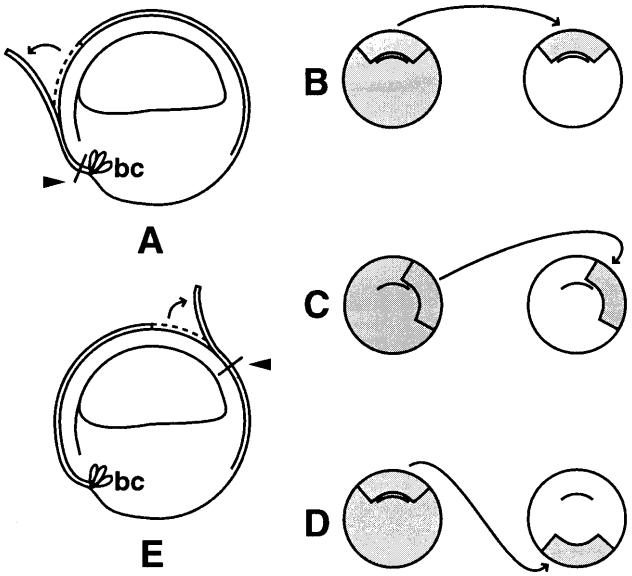

Fig. 1A–E Epithelial grafts of *Xenopus*. **A** Cutaway sagittal view of stage 10 embryo with dorsal marginal zone (DMZ) epithelium being removed (*arrow*). Dorsal is *left*. *Pointer* indicates location of vegetal cut (*bc* bottle cells of dorsal blastopore lip). **B–D** Vegetal views of marginal zone grafts. *Short arcs* indicate dorsal lips. Fluorescently labeled tissue is *shaded*. *Arrows* indicate transplantation of tissue from labeled donors (*left*) into unlabeled hosts (*right*). **B** Homotopic dorsal (DMZ) graft. **C** Homotopic lateral (LMZ) graft. **D** Heterotopic dorsal to ventral graft. **E** Removal of ventral animal cap epithelium for homotopic grafting

Results

Fluorescent SMZ grafts in *Xenopus* contribute cells to notochord and somites

In order to map the fates of superficial marginal zone (SMZ) cells in *Xenopus*, we homotopically grafted SMZ from fluorescently labeled early gastrula donors (stages 10–10.5) into unlabeled hosts of the same stage (Fig. 1A–C), allowed the grafted embryos to heal and develop, and fixed and sectioned them at various stages to reveal the locations of labeled cells, just as in our previous study with *Hymenochirus* (Minsuk and Keller 1996). In the earlier study, a spread of stages was analyzed, demonstrating that mesoderm invasion in *Hymenochirus* began at stage 15 and gradually progressed through stage 19, after which the surface-derived cells could be found in their final disposition in the mesoderm. Here the goal is to determine whether *Xenopus* undergoes a similar process at comparable stages. We therefore fixed grafted *Xenopus* embryos at tailbud stages (20–26) to determine whether labeled mesoderm cells are present, indicating that mesoderm invasion occurred prior to that time. We fixed additional *Xenopus* embryos at late gastrula/early neurula stages (11–14) to distinguish between delayed, stage-specific invasion (that occurring between stages 15 and 19 as in *Hymenochirus*) and early, non-stage-specific invasion (that occurring shortly after grafting). This distinction is important because early mesoderm invasion could be regarded as evidence of artifactual cell behavior caused by the grafting itself, not representative of behavior during normal development. Such an artifact is unlikely to manifest itself later, after complete healing of the graft followed by normal gastrulation.

Furthermore, labeled mesoderm cells found shortly after grafting might not indicate invasion at all, but rather the unintended transfer of labeled deep cells with the graft; the embryos fixed at late gastrula stage therefore serve as controls by demonstrating that the grafted epithelium was transferred cleanly without attached deep cells. There were occasionally a few labeled deep cells in such controls (typically 1–2), so this is considered a background level of contamination, and embryos were not scored positive for mesoderm invasion unless the number of labeled deep cells present was substantially higher.

Dorsal grafts

Twenty-one grafts were performed, and all the grafted embryos developed normally and were analyzed (Fig. 1A,B; Table 1). In nine embryos fixed as controls at late gastrula (stages 11.5–13), all labeled tissue was superficial (Fig. 2A) aside from a few stray cells in two of

Table 1 *Xenopus* epithelial grafting experiments

Type of graft	Stage fixed	Total number of grafted embryos scored	Number of grafted embryos scored positive for labeled cells in:		
			Notochord	Somite	Deep layer
Dorsal	11.5–13	9	0	0	
	20–26	12	9	2	
Lateral	12.5–14	3	0	0	
	22–26	9	0	5	
Dorsal to Ventral (heterotopic)	11–13	5			0
	22–24	3			3
Animal Cap	21–23	10			0

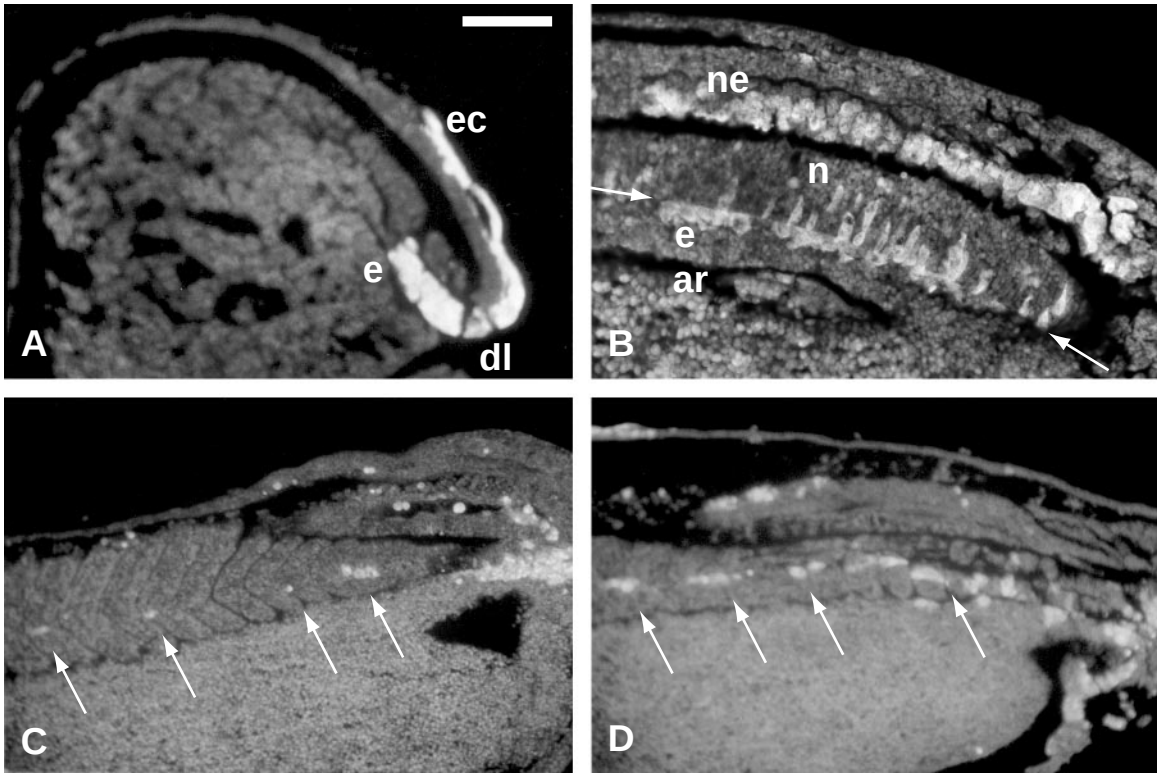

Fig. 2A–D Epithelially grafted *Xenopus* embryos. Sagittal sections; anterior *left*; dorsal *up*. **A** DMZ grafted embryo fixed at late gastrula (stage 12.5). Labeled epithelium is continuous around the dorsal lip of the blastopore (*dl*), in the process of involuting. Involuted regions occupy the superficial layer of the archenteron roof and give rise to endoderm (*e*), while non-involuting regions give rise to ectoderm (*ec*). No deep cells are labeled. **B** DMZ grafted embryo fixed at the tailbud stage. Labeled cells are seen in the neural tube (*ne*), the notochord (*n*), and the endodermal (*e*) roof of the archenteron (*ar*). *Arrows* indicate the boundary between notochord and endoderm. **C,D** Grafted embryos showing labeled cells in the posterior somites (*arrows*). **C** DMZ graft. **D** LMZ graft (*bar in A* represents 200 μm in **A, C** and **D**, 100 μm in **B**)

the embryos. Therefore only background levels of deep cells were transferred with the graft, and no non-stage-specific invasion by superficial cells occurred.

Twelve embryos were fixed at tailbud stages (Table 1). Nine of them contained substantial numbers of labeled notochord cells, well above background level (Fig. 2B), with typical stack-of-coin notochord morphology. Therefore, superficial cells can invade the notochord in *Xenopus*, specifically during neurulation, as in *Hymenochirus*.

In contrast to the consistent degree of mesodermal labeling in *Hymenochirus* grafts (Minsuk and Keller 1996), the degree of labeled cell contribution to *Xenopus* notochord varied widely. Four embryos contained over 50 labeled notochord cells each. Another 3 embryos contained between 20 and 30 labeled notochord cells each, while another contained 16 and 1 contained only 7, barely above the background level.

Only 2 of the 12 tailbud-stage embryos contained labeled somite cells (7 and 10 cells, respectively; Fig. 2C). In all 12 embryos, labeled tissue remaining in the superficial layer ended up in the midline, in contact with notochord but not with somites. Therefore, the paucity of labeled somitic tissue could reflect merely the position of the grafts, rather than any inability of superficial cells to invade the somites. We therefore performed lateral grafts in order to distinguish these possibilities.

Lateral grafts

Twelve lateral grafts were analyzed (Fig. 1C, Table 1). In 3 control embryos fixed as late gastrulae (stages 12.5–14), all label was in the superficial tissues. In 9 embryos fixed as tailbuds, none had any labeled notochord, but 5 had intermediate numbers of labeled somite cells (Fig. 2D), ranging from 11 to 19 each. These cases establish that *Xenopus* superficial cells can invade the somite. Most of the labeled cells were in posterior somites or in the unsegmented somitic mesoderm caudal to the youngest somite.

Heterotopic dorsal to ventral grafts

The experiments just described duplicate the same stage-specificity of invasion seen in *Hymenochirus* (Minsuk and Keller 1996), tending to rule out abnormal effects due to grafting as an explanation of this cell behavior. However, in previous work from this laboratory, Shih and Keller (1992) performed similar experiments, grafting heterotopically from the dorsal to the ventral side in an investigation of the organizing potential of the *Xenopus* DMZ epithelium. The grafts were made by different methods, and while that study also reported invasion of the deep layer, it was concluded that abnormal effects of grafting were the cause. Since it is important in the present study to rule out any such abnormal effects, we have repeated this experiment using our current methods (Fig. 1D). No non-stage-specific mesoderm invasion was seen (Table 1). No labeled deep cells were found in any of five grafted embryos fixed as late gastrulae, while labeled ventral mesoderm (scored simply as "deep layer" in Table 1 since ventral mesoderm is still undifferentiated at this stage) was found in three embryos allowed to develop to tailbud stage. Therefore, abnormal effects of surgery are not a likely explanation for the later movement of superficial cells into the mesoderm.

Animal cap grafts

Another way to distinguish normal from artifactual cell movements is that artifactual ones should be non-*region*-specific, occurring anywhere a graft is performed in the embryo. We therefore tested the region specificity of the deep layer invasion by performing animal cap grafts. Since the normal fate of the stage 10 animal cap is to end up as anterior ventral and lateral epidermis (Keller 1975, 1976), we investigated whether superficial animal cap cells invade the deep layer of the epidermis.

Grafts were performed as described above except that epithelium was taken from the ventral half of the animal cap of the donor, and transplanted homotopically to the corresponding location (and orientation) in the host (Fig. 1E). Since this region of the embryo remains accessible throughout gastrulation, additional grafts were performed as late as stage 12; results were consistent in all embryos regardless of the stage at which grafting was performed.

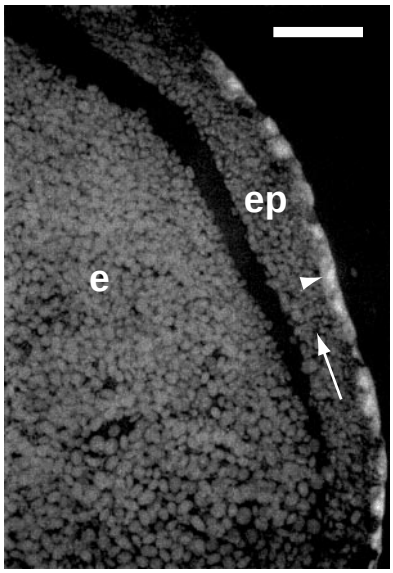

Fig. 3 Animal-cap-grafted *Xenopus* embryo, tailbud stage, sectioned transversely at mid-trunk level. Dorsal is *up*; mid-flank is shown, with somites out of view just beyond the top of the figure. Even at this late stage all labeled cells are in the superficial layer of the epidermis (*pointer*; *e* endoderm, *ep* epidermis, *arrow* deep layer of the epidermis, *bar* 100 μm)

Ten animal cap grafts were performed and all grafted embryos were allowed to develop to the tailbud stage (Table 1). The labeled grafts were found in the ventral and lateral epidermis, as expected. Not one embryo had any labeled cells in the deep layer (Fig. 3). This demonstrates that homotopically grafted animal cap epithelial cells do not invade the deep layer as do DMZ epithelial cells. As in *Hymenochirus*, deep layer invasion in *Xenopus* is both stage-specific and region-specific, and therefore is not a grafting artifact.

A protocol for fate mapping the superficial layer using cell surface biotinylation

In order to fully rule out surgical effects as a cause of mesoderm invasion, we used cell surface biotinylation to label epithelial cells without mechanically disrupting the tissue. Non-specific biotinylation of cell surface proteins (Hurley et al. 1985; Cole et al. 1987) makes it possible to label the superficial epithelial cells (apical surfaces only) without labeling deep cells (Lisanti et al. 1988; Choi et al. 1990). Biotinylation is non-toxic and does not impair protein function or cell behaviors in a variety of living systems studied (Flaherty and Swann 1993; Hoffmann-Fezer et al. 1993; Levy-Toledano et al. 1993; Tambourgi et al. 1993), including in clinical use with human patients (Cavill et al. 1988). Our viability controls establish that biotinylation is also non-toxic to *Xenopus* and *Hymenochirus* (see Materials and methods).

Biotin residues can be detected using a variety of conjugated avidins or streptavidins, proteins with extremely strong specific affinity for biotin, similar to that of antibod-

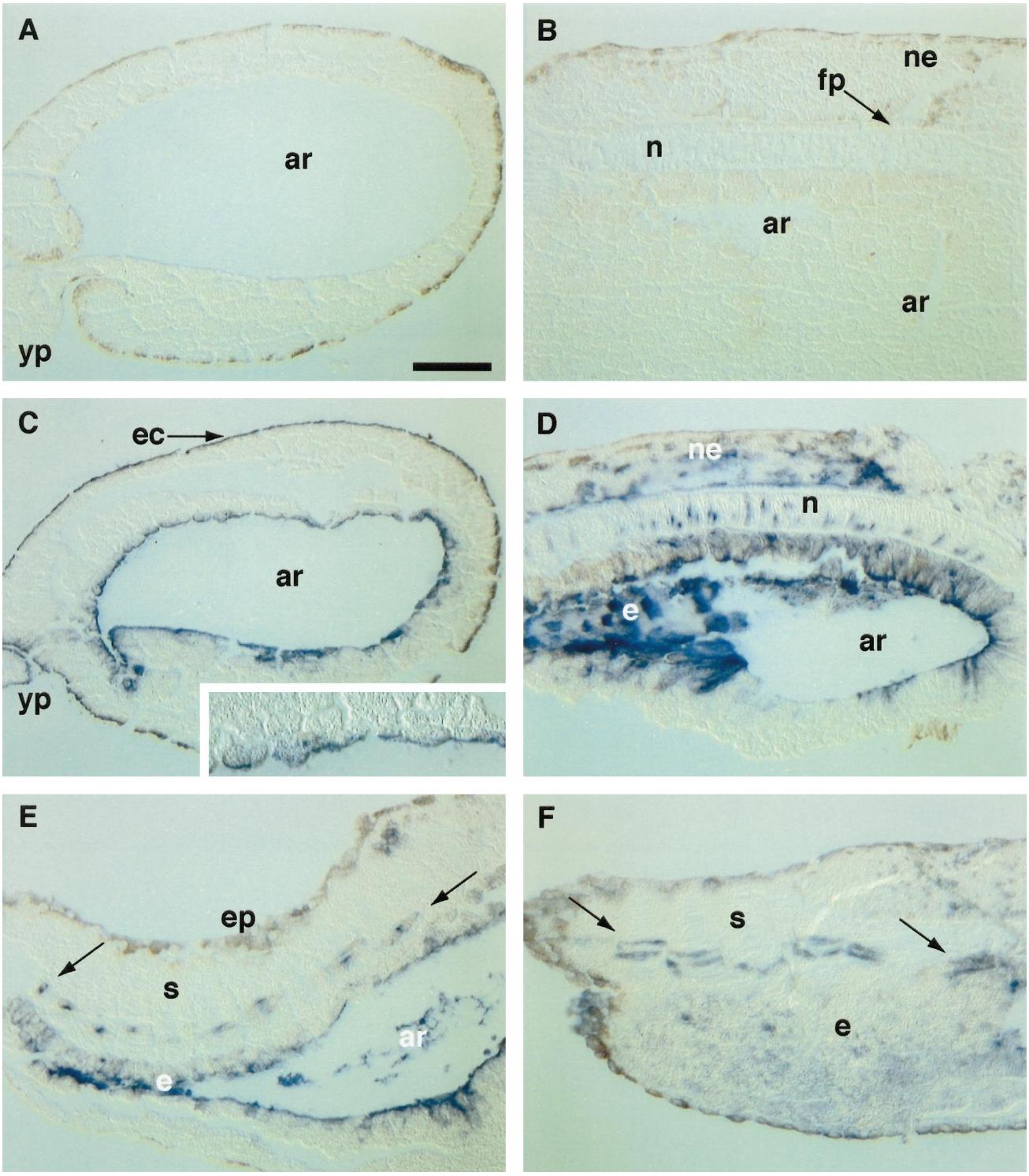

Fig. 4A–F *Hymenochirus* embryos, sectioned sagittally. Dorsal is *up*, anterior is *right*. **A** Unlabeled embryo at stage 14. Dark exterior edge is the embryo's natural pigment, which is darkest anteriorly and ventrally. No reaction product can be seen (*ar* archenteron, *yp* yolk plug). **B** Unlabeled tailbud stage embryo. No reaction product is present in any tissue. The embryo's natural pigment is visible in the dorsal ectoderm and in the lumen of the neural tube (*ne*. *fp* floor plate of the neural tube, *n* notochord). **C** Biotinylated embryo at stage 14. The entire lining of the archenteron as well as the surface of the ectoderm (*ec*) and the yolk plug are labeled with a dark blue precipitate. No precipitate is present in the deep cells. Gaps within the tissues are artifacts due to breakage. *Inset*: higher magnification of the archenteron roof, showing that precipitate is present only on the superficial cells (and in fact mostly on their apical surfaces). **D–F** Biotinylated embryos at tailbud stage. **D** Section through the notochord. The neural tube and endoderm (*e*) are heavily labeled, and individual labeled cells can be seen scattered along the length of the notochord. **E,F** Sections through the somites (*s*). Dorsal epidermis (*ep*) and endoderm (*e*) are labeled, and one (**E**) or more (**F**) stripes of labeled somite cells run along the anterior-posterior axis of the trunk. *Arrows* indicate the anteriormost and the posteriormost labeled somite cell in each section (*bar in A* represents 128 μm in **A** and **C**, 50 μm in **C** inset, 100 μm in all others)

Table 2 Biotinylation experiments

[a] Values for tailbud stage *Xenopus* are reported for 8 separate spawnings; values for early neurulae (from 7 of the 8 spawnings) and for *Hymenochirus* (2 spawnings) are cumulative. For tailbud stages of both species, notochord scores and somite scores for the same individuals are listed separately
[b] N=total number of embryos scored, or, in the case of somites at the tailbud stage, total numbers of flanks (sets of all the paraxial mesoderm along one side of an embryo, left or right; includes somites as well as posterior unsegmented mesoderm)
[c] Mean number of labeled cells detected per notochord, or per paraxial (somitic and posterior) stripe. Means are calculated using only positive-scoring notochords or flanks (those containing at least one labeled cell)
[d] Spawnings 4 and 5 were fertilized simultaneously using the same testis, and are therefore half-sibs

Species Stage (tissue):	Spawning no.[a]	N[b]	Number containing labeled mesoderm	%	Mean labeled cells each[c]
H. boettgeri					
13–14:		8	0	0	–
23–25 (notochord):		23	23	100	13.0
23–25 (somite):		45	45	100	9.0
X. laevis					
13–14:		18	0	0	–
21–30 (notochord):					
	1	7	0	0	–
	2	14	4	29	7.5
	3	10	0	0	–
	4[d]	10	2	20	4.5
	5[d]	10	1	10	1.0
	6	20	17	85	10.3
	7	6	6	100	25.8
	8	13	13	100	15.9
21–30 (somite):					
	1	11	0	0	–
	2	28	9	32	6.0
	3	20	0	0	–
	4[d]	20	0	0	–
	5[d]	20	0	0	–
	6	40	32	80	5.0
	7	12	12	100	11.0
	8	26	26	100	5.3

ies for their antigens (Hurley et al. 1985; Cole et al. 1987). We devised a protocol using alkaline phosphatase-conjugated streptavidin for the in situ visualization of the biotin signal in sectioned material (see Materials and methods).

Biotin labeling in *Hymenochirus* confirms the presence of surface notochord and somite

Unlabeled as well as biotin-labeled *Hymenochirus* embryos were fixed at early neurula and tailbud stages, sectioned, and processed for biotin visualization. Sections of unlabeled embryos remained uniformly devoid of precipitate (Fig. 4A,B), indicating that there was no background labeling whatsoever, due either to the presence of endogenous biotin, to the non-specific binding of streptavidin, or to the activity of endogenous phosphatases. All blue precipitate in the biotinylated embryos therefore indicates the presence of biotinyl groups introduced by the labeling procedure.

A total of eight labeled embryos in two spawnings (see Table 2) were fixed as early neurulae (stage 13–14), and a distinctly localized blue precipitate formed in all of them, in all superficial tissues both involuted and non-involuted: the superficial layers of the epidermis and neural plate as well as the lining of the archenteron and the surface of the yolk plug (Fig. 4C). Label was sharply restricted to the superficial cells; deep cells were not labeled anywhere (Table 2). This indicates that the biotinylation reaction was successfully restricted to the superficial cells of the stage 10 embryos, and that the covalently-bound label subsequently remained fixed to those cells and was not transmitted to deep cells. This establishes the validity of cell surface biotinylation for fate-mapping the superficial layer. Labeled deep cells seen in older embryos (see below) have therefore invaded the deep layer from the surface after stage 14.

Tailbud-stage embryos were scored by counting the number of labeled cells in the notochord or somites. Labeled mesodermal cells were distinctly marked with blue precipitate (Fig. 4D–F). Since the controls (early neurulae, Fig. 4C) were uniformly devoid of label in the mesoderm, even a few such labeled cells in the mesoderm of an older embryo are significant. The right and left flanks of each embryo were scored separately for labeled somite cells. In a few cases, the notochord or the somites on one flank of an embryo were unscoreable due to damage, abnormal development (a single embryo lacked a notochord), or localized artifactual labeling of the deep tissue (most likely due to damaged epithelium during the labeling procedure); Table 2 indicates total numbers of notochords scored and of somite flanks scored.

A total of 26 labeled embryos were fixed at tailbud stage and sectioned sagittally. All of them showed darkly labeled cells in the notochord as well as in the somites of both flanks. Labeled notochord cells were scattered throughout the notochord and had typical notochord cell morphology (Fig. 4D). Twenty-three notochords were scored (Table 2), containing 4–20 labeled cells each, with a mean of 13 labeled cells per notochord. We esti-

mate that a *Hymenochirus* notochord contains about a hundred cells, so that the superficial contribution ranges up to 20% in some individuals.

Labeled somite cells occurred in a consistent pattern, aligned at the same position in each somite and with normal somite cell morphology, resulting in a blue stripe from anterior to posterior (Fig. 4E). Often a double stripe was formed (Fig. 4F). Forty-five flanks were scored (Table 2), each containing a stripe across 2–15 somites, with a mean of 9 somites per stripe. The surface contribution to somites is harder to quantify than that of notochord, but is probably somewhat less, perhaps 5%, based on the areas of successive serial sections found labeled.

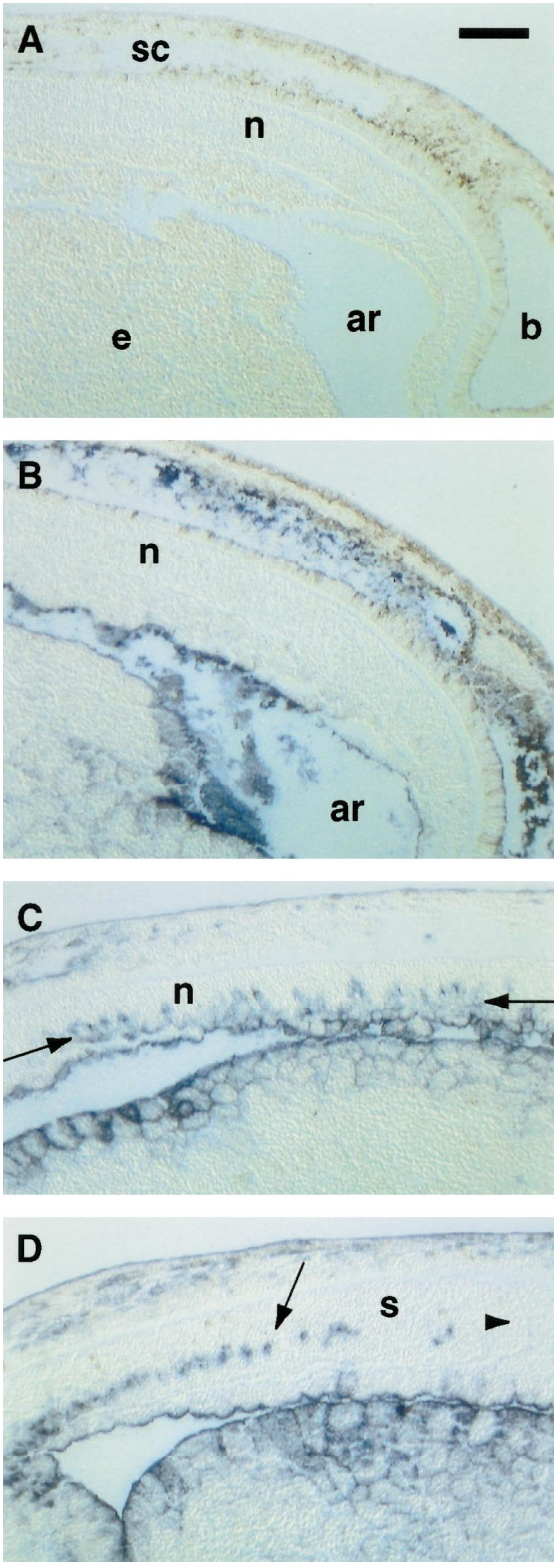

Fig. 5A–D *Xenopus* embryos, stage 22, sectioned sagittally. Dorsal is *up*, anterior is *right*. **A** Unlabeled embryo. The embryo's own pigment appears brown in the epidermis and in the neural tube (*b* brain, *sc* spinal cord). No reaction product is present (*ar* archenteron, *e* endoderm, *n* notochord). **B** Similar section of a biotinylated embryo. The same structures are visible, but dark blue precipitate can be seen lining the archenteron, and in the neural tube. In this embryo, there is no trace of precipitate in the notochord. **C** A biotinylated embryo similar to **B**, but in this case notochord cells are labeled. *Arrows* indicate the boundary between notochord and endoderm. **D** A biotinylated embryo showing labeled posterior paraxial mesoderm (*arrow, s* somitic mesoderm, *pointer* segmentation boundary of most posterior somite, *bar* 100 μm)

Biotin labeling in *Xenopus* reveals variable amounts of presumptive mesoderm in the superficial layer

We performed the identical experiment in *Xenopus* embryos. As in *Hymenochirus*, unlabeled embryos were completely devoid of precipitate (Fig. 5A), indicating complete absence of background labeling. Also as in *Hymenochirus*, embryos fixed as early neurulae (stage 13–14) contained precipitate only in superficial tissues, and never in deep cells (Table 2).

As above, tailbud-stage embryos were scored by counting labeled mesoderm cells, and each flank of an embryo was scored separately for somitic mesoderm. Notochord and somitic mesoderm both contained labeled cells, but there was great variation between spawnings, both in the percentage of embryos containing labeled mesoderm, and in the number of labeled mesoderm cells in each embryo (Table 2). Labeled notochord cells were found at all antero-posterior levels (Fig. 5C), but in contrast to *Hymenochirus*, labeled somitic mesoderm was found only in the most posterior region; anterior and mid-trunk somites were never labeled (Fig. 5D). Since most of the embryos were scored prior to stage 24, before somite segmentation or rotation has reached the posterior mesoderm, labeled paraxial mesoderm cells did not yet display the distinct somitic morphology as did those in *Hymenochirus*, and were more difficult to count. Variations in the intensity of mesodermal label also introduced difficulty. Therefore, as in the grafting experiments, the values reported in Table 2 are low estimates.

A total of 90 tailbud-stage notochords were scored, and labeled cells were found in 43 of them (48%). These ranged from 1 notochord containing only a single detectable labeled cell, to 1 containing at least 35 labeled cells, with a mean of 13.4 labeled cells detected per positive-scoring notochord. These results are broken down by

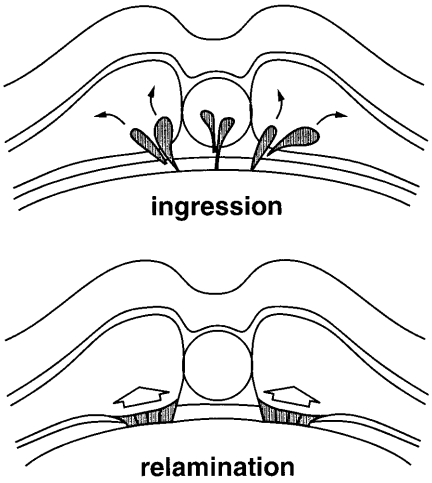

Fig. 6 Alternative mechanisms of mesoderm invasion by surface cells. *Top* The ancestral mode of invasion in anurans is ingression, the individual detaching of surface cells from one another and their independent entry into the mesoderm. *Bottom* In relamination, superficial cells dissociate from neighboring epithelia en masse (without dissociating from one another) and associate instead with the mesoderm. (After Minsuk and Keller 1996)

spawning in Table 2, demonstrating the large variation (Fig. 5B,C). We estimate that a *Xenopus* notochord contains a few hundred cells, so that the number of labeled cells reported here, while similar to that in *Hymenochirus*, represents a smaller surface contribution, as high as 10%.

Somites and posterior unsegmented paraxial mesoderm were scored in a total of 177 embryonic flanks. Labeled cells were detected in 79 of them (45%), forming a stripe about a third to a half of the way up from the ventral boundary of the mesoderm (Fig. 5D). These were restricted to the posterior portion of the embryo (a restriction not present in *Hymenochirus*), and varied from only a few labeled cells in the most posterior tailbud mesoderm, to as many as 20, with a mean of 6.1 labeled cells per positive-scoring paraxial stripe (Table 2).

Morphogenetic mechanism

In a few cases, serial sections of labeled *Xenopus* embryos provided evidence for the morphogenetic mode of cell movement from the superficial to the deep layer. Previously (Minsuk and Keller 1996), we described relamination in *Hymenochirus*, an unusual morphogenetic mechanism in which paraxial superficial cells maintain their epithelial organization and their continuity with neighboring epithelia as they collectively form an association with the deep layer, and subsequently become covered by endoderm migrating in laterally. In contrast, surface mesoderm in other anurans invades by ingression of individual cells (Purcell 1992; Purcell and Keller 1993). In relamination, therefore, one finds intact epithelia interacting with the deep layer as a whole, while in ingression one finds individual cells migrating from one layer to the

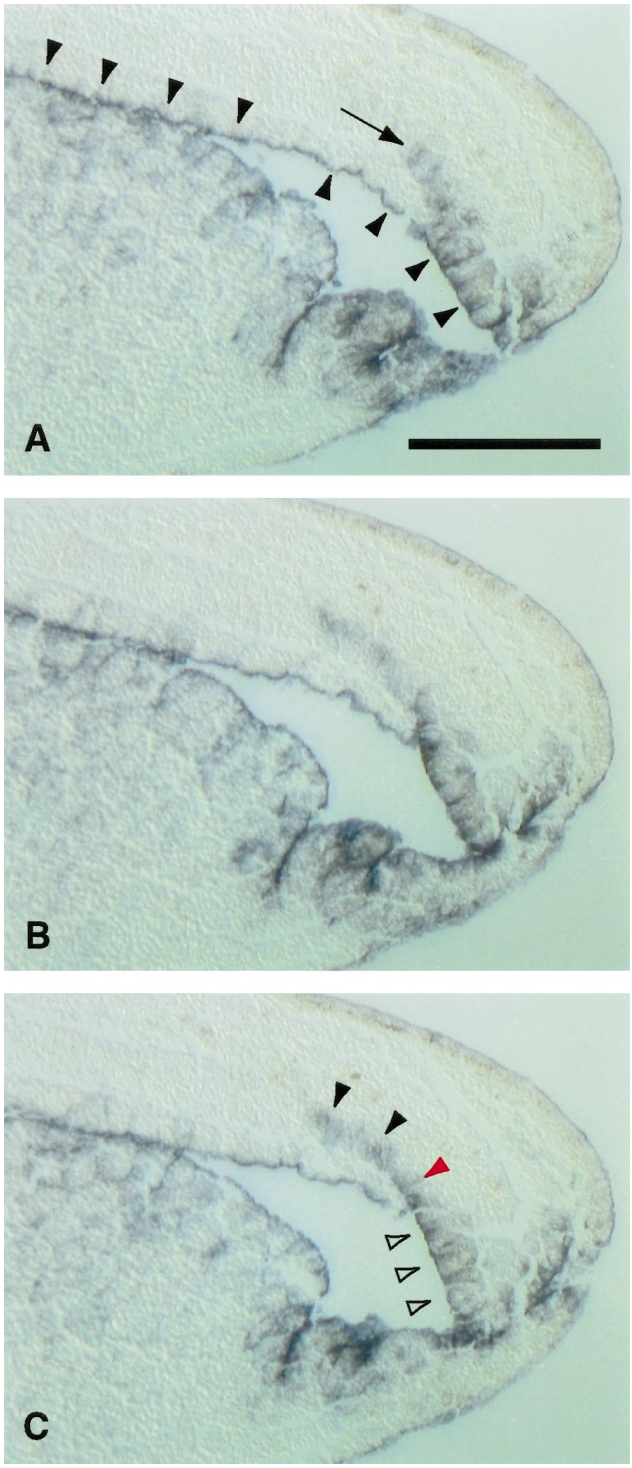

Fig. 7A–C Three adjacent sagittal sections through the posterior paraxial mesoderm of a stage 22 biotinylated *Xenopus* embryo. Dorsal is *up*. **A** Most lateral section; **B** middle section; **C** most medial section. The arrangement and morphology of the labeled cells suggests that superficial cells invade the deep layer by relamination rather than by ingression. See text for details. (*Bar* 200 μm)

other, identifiable by their reduced apical surface area and associated bottle cell shape (Fig. 6). Our data suggest that *Xenopus* paraxial surface mesoderm invades by relamination.

Figure 7 shows a series of three adjacent sections through the posterior somitic mesoderm of a biotin-labeled *Xenopus* embryo fixed at tailbud stage (stage 22). Figure 7A is the most lateral section. As usual the inner surface of the archenteron roof is labeled with blue precipitate (pointers). A few posterior deep cells are also labeled (arrow), indicating their superficial origin. The archenteron roof surface is a coherent, continuous layer, and the labeled deep cells are part of a clearly distinct deep layer. In the most medial section (Fig. 7C), the most posterior part of the labeled epithelium (open pointers) is no longer continuous with the epithelium just anterior to it, but is continuous instead with the labeled deep cells in the adjacent layer (solid pointers). Most of these deep cells have a more diffuse labeling, characteristic of mesenchymal cells, but those closest to the surface (solid red pointer) retain the sharp-edged labeling of epithelial cells. The intervening section (Fig. 7B) has intermediate morphology and demonstrates the continuity of the labeled cells in the three sections. This arrangement of tissues is similar to that seen in *Hymenochirus* using scanning electron microscopy (SEM; Minsuk and Keller 1996), and provides preliminary evidence that when *Xenopus* superficial cells invade the somitic mesoderm, they do so by relamination as does *Hymenochirus*.

Discussion

The grafting experiments performed here indicate that labeled *Xenopus* SMZ cells have the ability to invade the deep layer. Dorsally grafted cells invade the notochord, while laterally grafted cells invade the somite. This occurs only after stage 14, long after the grafts have healed in. Animal cap grafts do not contribute any cells to the deep layer by the early tailbud stages, indicating that the behavior is restricted to the marginal zone. This stage and region specificity, parallel to that in *Hymenochirus*, argues against surgical disruption or destabilization as the cause of these movements.

Our new method for superficial fate mapping using cell surface biotinylation confirms and extends this result. The presence of both surface notochord and surface somite cells in *Hymenochirus* is consistent with our earlier demonstration (Minsuk and Keller 1996) of stage-specific invasion between stages 15 and 19.

The same method applied to *Xenopus* demonstrates that even in the absence of surgical disruption of any kind, superficial cells often invade the deep layer and contribute to notochord and posterior somitic mesoderm. The invasion takes place by stage 21 but only after stage 14, paralleling the behavior of fluorescently grafted tissue as well as the stage specificity seen in *Hymenochirus*. Similarly, mesoderm invasion in *Rana pipiens* also takes place during neurulation, as shown by both non-surgical and surgical methods (Delarue et al. 1994, 1996).

Labeled somitic cells form a remarkably consistent horizontal stripe pattern under different conditions and in both species (compare Figs. 2C,D, 4E,F and 5D). This may simply reflect the origin of the invading cells at a consistent time and place, combined with the predictable subsequent morphogenetic transformations of the somitic mesoderm; it remains an open question whether the pattern has functional significance. Surface-derived cells could maintain distinct identities and have distinct fates in later development, but this is unlikely given the variable length of the stripes and the frequent occurrence in *Hymenochirus* of double stripes (Fig. 4F).

The extensive similarities of surface mesoderm invasion in both species and under both experimental approaches (the somitic stripe pattern, the stage specificity of invasion, and the region specificity revealed by the grafting experiments) suggest common mechanisms of temporal and spatial regulation and a homology of process in the two species.

The evidence against surface mesoderm in *Xenopus*

Our results challenge the established belief that all mesoderm in *Xenopus* arises in the deep layer. The evidence for this belief has never been airtight. Nieuwkoop and Florschütz (1950) examined *Xenopus* in histological sections, and rejected deep layer invasion by surface cells because they saw no evidence of mass tissue movements. But they described the major features of mesoderm morphogenesis only through stage 13, focussing solely on neural development in later stages. As we have shown, the superficial contribution is not made until at least stage 15. In addition, they dismissed on theoretical grounds the possibility that invasion might occur by individual ingression of small numbers of scattered surface cells, undetectable by their methods.

Two subsequent studies used surface labeling methods to map the normal fate of the epithelial cells, and while both studies concluded that mesoderm receives no surface contribution, both studies also reported at least some exceptions. Vital dye staining (Keller 1975) revealed a few cases of superficial dye marks going deep, but too few to distinguish this from any effects of overstaining; the possibility of a small and perhaps variable amount of surface mesoderm in *Xenopus* was left open. Smith and Malacinski (1983) are often cited (Lundmark 1986; Elinson 1987; Delarue et al. 1992, 1994; Shih and Keller 1992; Saint-Jeannet and Dawid 1994) for their statement that “superficial cells of *Xenopus laevis* blastulae do not contribute to the mesoderm,” but a closer reading of that paper reveals that the ventral part of the stage 19–21 notochord was labeled in half of their specimens, indicating superficial contribution. Convincing controls, similar to those in this study, re-

vealed no labeled deep cells at stages 9–13, after labeling performed at stage 9, so subsequent labeled notochord can only be explained by surface cell invasion. The discrepancy between their data and their conclusion was not explained.

Finally, other studies have demonstrated that *Xenopus* SMZ cells are competent to form mesoderm, consistent with a role in normal mesoderm development. Nieuwkoop reconsidered the possibility of individually ingressing surface cells in normal development upon showing (Sudarwati and Nieuwkoop 1971) that surface cells can be induced to differentiate as notochord and muscle, and do so with vigor in animal/vegetal recombinates. In grafting experiments similar to those performed here (Shih and Keller 1992), superficial cells invaded the deep layer and contributed to mesodermal structures. Surface cells deliberately placed into the deep tissue (Shih and Keller 1992) were able to participate in normal axial mesoderm development. Here, we show that this potential is often realized in normal development.

Developmental alternatives

This study reveals a great deal of between-spawning variation in the origin of surface mesoderm in *Xenopus*. Such qualitative variation, in which an entire developmental pathway can be either present or absent in different individuals of the same species, is unusual for commonly studied developmental phenomena. Phenotypic polymorphisms within populations are common in adult characters (Futuyma 1986), and even in larval morphology (Greene 1989; Pfennig 1992), but the presence of alternative developmental pathways in very early embryos challenges long-standing notions of developmental conservatism and canalization (von Baer 1828, partially translated in Henfrey and Huxley 1853; Arthur 1988). Developmental diversity between species is commonplace (Elinson 1987; Henry and Raff 1990; Wray and Raff 1990; Purcell and Keller 1993; Collazo et al. 1994; Minsuk and Keller 1996; Sommer and Sternberg 1996), and the same may prove true of developmental alternatives within species. For example, in amniotes there is an ontogenetic polymorphism not only within species but within individuals: the trunk neurulates by fusion of the neural folds, but the tail neurulates by cavitation of a solid rod (Schoenwolf and Delongo 1980; Schoenwolf 1984). In the sea urchin *Strongylocentrotus purpuratus*, in which vegetal plate specification in macromere derivatives is induced by a signal from the micromeres, the duration of micromere/macromere contact required for successful signaling varies greatly between spawnings (Ransick and Davidson 1995). Polymorphisms in development can occur at the molecular level as well, as recently shown for the *Drosophila Ultrabithorax* gene (Gibson and Hogness 1996).

There are many ways to make mesoderm (Brun and Garson 1984; Lundmark 1986; Gajović et al. 1989; Purcell and Keller 1993; Sulik et al. 1994; Minsuk and Keller 1996), and it appears that *Xenopus* can avail itself of two of them. The developmental "program" for *Xenopus* dorsal mesoderm thus does not progress in rigid lockstep, but with flexibility, able to express variation at one stage without altering or impairing later development. Studies of molecular or cellular processes involved in organizer function or mesoderm morphogenesis and patterning must take this variation into account.

Evolution of mesoderm invasion

The presence of surface mesoderm is ancestral and general for anurans (Purcell 1992; Purcell and Keller 1993). Previously we demonstrated (Minsuk and Keller 1996) that this feature is present even in a near relative of *Xenopus*, and, under the assumption that it is absent in *Xenopus* and given the phylogenetic position of *Xenopus*, we proposed that surface mesoderm was lost in the *Xenopus* lineage only after the divergence of *Xenopus* from *Hymenochirus*. We must now reformulate this to state that mesoderm invasion became facultative in the *Xenopus* lineage after that divergence. The discovery of surface mesoderm in at least some *Xenopus* individuals provides an opportunity to test our evolutionary scenario.

Hymenochirus surface mesoderm invades the somites by relamination (Minsuk and Keller 1996). The more ancestral anuran mode of invasion by independent ingression of individual cells produces different cell morphologies that can be distinguished from those of relamination using SEM (compare Purcell and Keller 1993; Minsuk and Keller 1996). We proposed that relamination may represent an evolutionary intermediate in the loss of surface mesoderm in *Xenopus*, and would have been present in the *Hymenochirus*/*Xenopus* common ancestor (Minsuk and Keller 1996). Given that surface mesoderm is in fact present in *Xenopus*, we reformulate this proposal as follows: relamination may represent an evolutionary precursor to the subsequent evolution of intraspecific variation. This can be tested by examining the morphology of invading *Xenopus* mesoderm. If our scenario is correct, we would predict that any somite invasion in present-day *Xenopus* should occur by relamination. Invasion by ingression would falsify our scenario, implying that relamination arose more recently in the lineage leading to *Hymenochirus*, and never existed in any direct ancestors of *Xenopus*. SEM would allow superior views of fine cell and tissue morphology, but our paraffin sections (Fig. 7) offer preliminary evidence suggesting that *Xenopus* uses relamination, thus supporting our proposal that relamination was an intermediate stage in the evolution of facultative surface mesoderm invasion in *Xenopus*. Thus developmental data can be used not only to generate, but to test, evolutionary hypotheses.

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