

# Two distinct sources for a population of maturing axial progenitors

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In mammals, the primitive streak region and its descendant, the tail bud, are the source of nascent mesoderm and spinal cord throughout axial elongation. A localised population of long-term axial progenitors has been identified in a region of the tail bud, the chordoneural hinge, but the localisation of such progenitors at earlier stages is so far untested. By studying gene expression, we have shown that a specific topological arrangement of domains persists from the streak to the tail bud, and includes an area (the node-streak border) in which ectoderm that expresses primitive streak markers overlies the prospective notochord. This arrangement persists in the chordoneural hinge. Homotopic grafts show that, as in other vertebrates, cells in the streak and node predominantly produce mesoderm, whereas those in the node-streak border and lateral to the streak additionally produce neur ectoderm. Node-streak border descendants populate not only neur ectoderm, somites and notochord throughout the axis, but also the chordoneural hinge. Ectoderm lateral to the embryonic day (E)8.5 streak is later recruited to the midline, where it produces somites and chordoneural hinge cells, the position of which overlaps that of border-derived cells. Therefore, the E8.5 axial progenitors that will make the tail comprise cells from two distinct sources: the border and lateral ectoderm. Furthermore, heterotopic grafts of cells from outside the border to this region also populate the chordoneural hinge. Expression of several streak- and tail bud-specific genes declines well before elongation ends, even though this late population can be successfully transplanted into earlier embryos. Therefore, at least some aspects of progenitor status are conferred by the environment and are not an intrinsic property of the cells.

**KEY WORDS:** Mouse, Rostrocaudal axis, Axial elongation, Stem cells, Node, Primitive streak, Chordoneural hinge

## INTRODUCTION

Vertebrate axial elongation depends on a small population of axial progenitors (i.e. cells that produce differentiated derivatives along the anteroposterior axis) located at the caudal end of the embryo. In mammals and birds, these cells are located in and near the primitive streak and its descendant, the tail bud, and lay down the axis in a rostral-to-caudal sequence (reviewed in Stern et al., 2006). In the mouse, this process lasts for around 6 days, eventually producing some 60 somites – segmented blocs of mesoderm that contain progenitors of the axial skeleton and musculature. These flank the neural tube – the progenitor of spinal cord – and the notochord, a mesodermal tissue that patterns the neural tube and somites (Kaufman, 1992). Clonal lineage analyses in mouse and chick have indicated that at least some axial progenitors are present for long periods of axial elongation (here termed long-term axial progenitors), giving rise to descendants that contribute to differentiated axial tissues over large rostrocaudal distances (Mathis and Nicolas, 2000; Nicolas et al., 1996; Selleck and Stern, 1992a). Thus, these long-term axial progenitors might be stem cells (cells that can self-renew as well as differentiate). Consistent with these data, a series of transplantation experiments has suggested the presence of self-renewing cells in a region of the tail bud termed the chordoneural hinge (CNH) (Cambray and Wilson, 2002). When CNH cells were transplanted from embryonic day (E)10.5–12.5 (35–55-somite stage) tail buds into the primitive streak region of E8.5 (2–6-somite stage) embryos that were then cultured for 48 hours, they contributed to rostral somites, notochord and neural tube. Therefore,

progenitors of the caudal axis retain the capacity to produce more rostral axial tissues. Moreover, descendants of these cells were also retained in the CNH and could be passaged through successive embryos, and thus could repeatedly incorporate in the same segments of the axis. Together, these lineage analyses and transplantation studies provide evidence that at least some long-term axial progenitors are stem cells.

In the above transplantation studies, all donor tissues were grafted to the junction between the node (which gives rise to notochord) and the rostral primitive streak (which generates somites) to provide a standard environment to compare different graft types. In these experiments, grafting the node to the junction of the node and streak showed two distinct patterns of axial colonisation: some grafts gave rise exclusively to axial tissues (the ventral neural tube and notochord), whereas others additionally colonised the somites. The grafts that colonised somites also gave rise to cells in the tail bud that were subsequently transplanted for several generations. Although the number of grafts was too small to distinguish a significant effect, it suggested that grafts that included somite progenitors might contain a localised source of axial stem cells, whereas those that contained only notochord and neural tube did not.

Several features of the E8.5 mouse embryo make a more careful analysis possible. The node and rostral streak are initially not covered by the ventral part of the hindgut tube, and are therefore accessible to manipulation. Culture of E8.5 embryos is straightforward over the ensuing 48-hour period when around half of all somites are made (Copp and Cockcroft, 1990). Furthermore, at E8.5, the node is a morphologically distinct structure. It consists of a group of ventrally located notochord progenitors (Beddington, 1994; Wilson and Beddington, 1996) that are transiently inserted in the endoderm layer and are bounded laterally and caudally by a semicircular raised edge of radially oriented cells (Sulik et al., 1994) [elsewhere termed the ‘crown’ (Bellomo et al., 1996)]. The caudal

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aspect of this edge delineates the boundary between the node and primitive streak. This ventral region of the node and the ectoderm immediately above it form a smooth convex shape when viewed from the dorsal side. The junction with the streak forms an abrupt transition to the concave shape of the streak, and is thus morphologically distinct. Later, as the hindgut invaginates and the notochord separates from the endoderm layer between E8.5-E9.5, these morphological distinctions disappear.

In this study, we therefore investigated the gene expression and functional properties of axial progenitors in the node, the primitive streak and their environs at E8.5. Firstly, we carefully compared gene expression in the E8.5 primitive streak with that in the tail bud from E10.5-E13.5. This analysis revealed a striking similarity between the node-streak border at E8.5 and the CNH in the tail bud. However, we also found distinctions between early and late progenitors. Secondly, we investigated the fate and commitment to these fates of these different regions, and found that these are distinct and stereotyped. In particular, the node-streak border contains a high proportion of continuing long-term axial progenitors, whereas cells lateral to the streak are incorporated less prominently in the CNH.

## MATERIALS AND METHODS

### Maintenance of mouse stocks and culture of embryos

MF1 and *TgN(beta-actEGFP)04Obs* (Okabe et al., 1997) ('GFP transgenic') mice were maintained on a 14-hour light, 10-hour dark cycle. Noon on the day of finding a vaginal plug was designated E0.5. Dissection and culture was performed as described (Copp and Cockcroft, 1990)

### In situ hybridisation

Embryos, tails and tissue fragments were subjected to whole-mount in situ hybridisation as described previously (Wilkinson, 1992) except that proteinase K treatment was empirically adjusted between 5-18 minutes according to embryo size and stage. The riboprobes used were: *T* (brachyury) (Herrmann, 1991), *Fgf8* (Mahmood et al., 1995), *Cdx2* (Tanaka et al., 1998), *Wnt3a* (Takada et al., 1994), *Evx1* (Dush and Martin, 1992), *Foxa2* (Sasaki and Hogan, 1993) and *Sox1* (Aubert et al., 2003). Embryos were then dehydrated via an ethanol series, processed for paraffin wax histology and sectioned at 7 µm in a microtome (Anglia Scientific 0325).

### Embryo dissection and grafting

GFP transgenic × MF1 litters were dissected as described (Cambray and Wilson, 2002). The node and primitive streak areas were dissected using fine glass needles by making longitudinal lateral cuts, isolating two adjacent ~100 µm-wide strips of tissue containing either the entire midline – including the rostral node, border and streak 1-5 regions – or the area lateral to the streak – including the lateral border and lateral 1-5 regions (Fig. 1A).

The regions were further dissected by making transverse cuts with glass needles. Dissection of wild-type MF1 recipient embryos, grafts and culture were performed as described (Cambray and Wilson, 2002).

### Embryo processing

At the end of the culture period, green fluorescence was assessed in a Zeiss Stemi SV11 dissecting microscope with fluorescence attachment. Images were captured using Improvision Openlab software and processed using Adobe Photoshop. Grafted embryos and *Sox1-GFP* embryos were embedded in 10% gelatin; 10% albumin in PBS, fixed in 4% PFA, then coated with a 0.05% gelatin, 15% BSA; 20% sucrose; 10% glutaraldehyde solution to create a tear-resistant outer layer. Transverse sections were cut in a Series 1000 Vibratome at 100 µm and then incubated with TO-PRO-3 (Molecular Probes) for 30 minutes before being mounted under coverslips in Vectashield (Vector Laboratories) for immediate confocal microscopy.

### Assessing the contribution of GFP-labelled cells to axial tissues

In cases in which the contribution of cells from adjacent graft types (e.g. lateral regions 1,2,3 or streak regions 2,3,4) was indistinguishable, results were pooled for scoring in Table 1. To quantify the contribution of grafted cells in each tissue, the number of sections that showed integration in a given tissue was expressed as a proportion of the total number of sections that contained any labelled cells. Labelling in more than 50% of the sections was considered 'high level', corresponding to at least 10 out of a total of ~20 scorable sections. Integration in 20-50% of sections was considered 'low level'. Integration in less than 20% of the sections was scored as zero, because this usually corresponds to a small clump of cells in the area around the original graft site. The position of the tissue scored as 'CNH' is indicated in Fig. 1B. Mesenchyme caudal to the CNH was scored as tail bud mesoderm (TBM). In the tail bud, where there are relatively few sections, any integration was scored as high level for either CNH or TBM.

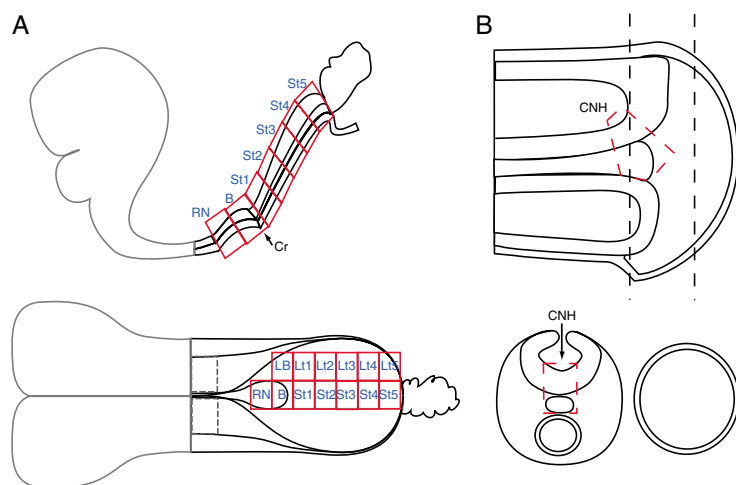
### Imaging

In vibratome sections, fluorescence was detected using laser excitation wavelengths of 488 nm for GFP and 633 nm for TO-PRO-3, and 500-600 nm (green) and 650-700 nm (red), respectively, for detection, which was carried out in a confocal microscope (Leica DM IRE2; Leica Microsystems). Images were captured using Leica Confocal Software v2.61 (Leica Microsystems) and noise filtered in a Workstation G5 using Volocity LE software (Improvision). Images were processed using Adobe Photoshop CS (Adobe Systems).

## RESULTS

### A topographical expression map of the primitive streak and tail bud

To precisely define the topological relationship between the primitive streak and tail bud (Cambray and Wilson, 2002), we performed a careful gene expression study on a selection of genes



**Fig. 1. Schematic representation of grafted and scored regions.** (A) Lateral (top) and dorsal (bottom) views of the primitive streak showing the regions dissected. The rostral end of the node (RN) was recognised by its wider notochordal plate, and the border (denoted by B) was recognised by the crown (cr) at the caudal end of the node. (B) Schematic diagrams showing the position of the CNH (red dashed lines) in sagittal sections (upper panel; as shown in Fig. 2 and in Fig. S1 in the supplementary material) and transverse sections (lower panel; as shown in Figs 5 and 6). Black dotted lines on the upper panel show the rostrocaudal position of transverse sections. CNH, chordeuroneural hinge

known to be expressed in the primitive streak and tail bud. Consistent with the expression patterns previously reported for these genes (Takada et al., 1994; Crossley and Martin, 1995; Herrmann, 1991; Kispert and Herrmann, 1994; Dush and Martin, 1992; Ang et al., 1993), we observed *Wnt3a*, *Fgf8*, *T* (brachyury) and *Evx1* expression in the E8.5 primitive streak and emergent mesoderm (Fig. 2A,C,E,G), and *Foxa2* and *T* expression in the ventral layer of the node (Fig. 2G,G',I,I'). *Cdx2* was expressed broadly in the streak and tail bud, encompassing all tissues in the streak and node at E8.5, as well as the entire CNH and tail bud mesoderm (TBM) at E10.5 (Fig. 3I and data not shown). However, somewhat surprisingly, the expression domain of *Wnt3a*, *Fgf8*, *T* and *Evx1* in the streak terminated not at the streak-node junction, but in the dorsal (ectoderm) layer of the caudal half of the node itself, overlying a domain of *Foxa2* and *T* expression in the ventral (mesoderm) layer (Fig. 2A',C',E',G',I'). The expression of *Wnt3a*, *Fgf8*, *T* and *Evx1* appeared to be complementary to a further *Foxa2* expression domain in the ectoderm of the rostral half of the node. An additional novel expression domain was seen at the caudal tip of the ventral node, which expressed *Fgf8* (Fig. 2A'). These expression domains were mirrored in the tail bud, because ectoderm cells expressing *Wnt3a*, *Fgf8*, *T* and *Evx1* overlaid cells in the ventral, emergent notochord that expressed *Foxa2* and *T*, and, at the caudal notochord tip, *Fgf8* (Fig. 2B,D,F,H,J). This region of apposed gene expression corresponded to the CNH, which we have previously shown contains long-term axial progenitors, raising the possibility that the node-streak junction at E8.5 contains similar axial progenitors.

*Sox1* expression was examined using in situ hybridisation and a GFP transgene targeted to the *Sox1* locus (Ying et al., 2003). GFP protein was found at high levels in E8.5 embryos in all neurectoderm as far caudally as the rostral half of the node (Fig. 2K and data not shown). Because *Sox1* is thought to be a neural-specific marker, it was somewhat surprising that it was detected at relatively high levels in the ectoderm of the CNH (Fig. 2L,N). It was also present at lower levels in the TBM (Fig. 2L',O), which we have previously shown to be the immediate descendant of CNH ectoderm (Cambray and Wilson, 2002). The expression of *Sox1* mRNA was essentially the same as that of GFP protein, except that *Sox1* mRNA was almost undetectable in the TBM (Fig. 2M), suggesting that expression is quickly downregulated on mesoderm formation, whereas GFP protein persists in this tissue. Intriguingly, cells strongly positive for *Sox1*-GFP were present in the caudal notochordal territory (Fig. 2N,N'), and some were continuous with two ventrolateral horns of the neural tube, suggesting that cells from the *Sox1*-GFP-positive neurectoderm can intermingle with notochord, at least at its caudal and lateral extremities. Thus, axial progenitors upregulate *Sox1* during their transition from primitive streak to tail bud.

To determine whether the progenitors remained unchanged during axis elongation, we also examined the expression of *T*, *Wnt3a*, *Fgf8* and *Cdx2* in E11.5-E13.5 tail buds. The late expression domains of genes in the tail bud were similar to those at earlier stages, confirming a similar topology of the tail bud as axial elongation progresses (Fig. 3 and see Fig. 1 in the supplementary material). However, at these late stages, the expression levels changed dramatically. *Fgf8* and *Wnt3a* were expressed strongly throughout the tail bud until E10.5 (Fig. 3A,E) but, thereafter, their expression declined (see Fig. S1 in the supplementary material). Analysis of sections shows that this is due to both a decrease in the number of expressing cells and in the levels of expression per cell. Expression essentially disappeared by E13.5, when elongation ceased (Fig. 3B-D,F-H'). Similarly, *Cdx2* expression was strong until E11.5 (Fig.

3I,J and see Fig. 1 in the supplementary material), at which point its expression declined, becoming undetectable by E13.5 (Fig. 3L). *T* transcripts were present at high levels until E12.5 (Fig. 3M-O and see Fig. 1 in the supplementary material), and were still detectable at lower levels at E13.5 (Fig. 3P). Thus, the expression of several genes declines 48 hours or less before the end of axis elongation.

### Fate mapping and commitment to given fates

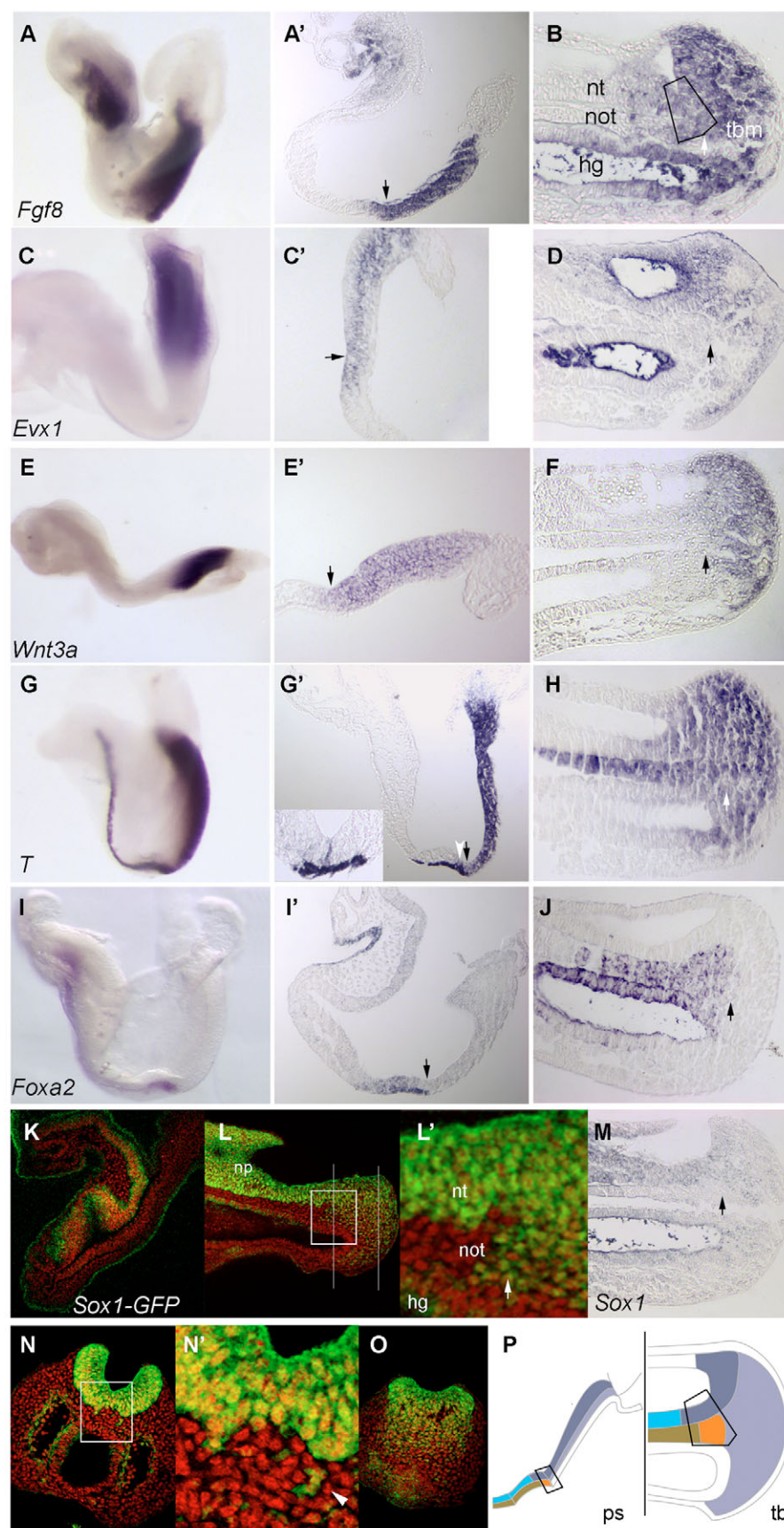
To examine the fate and commitment to these fates in the above regions, and in particular their relationship with later axial progenitors in the CNH, we manually microdissected regions of the E8.5 streak, node and lateral areas according to the scheme shown in Fig. 1. Thus, rostral and caudal regions of the node ('rostral node' and 'border', respectively, the latter containing the crown at the node-streak border), five successively more caudal pieces of the primitive streak ('streak 1-5'), and the regions lateral to the border and streak (lateral border, lateral 1-5 regions) (Fig. 1A, Fig. 4A-C) were isolated. To assess accurate dissection, rostral node, border and streak 1 pieces were subjected to in situ hybridisation for *Fgf8* or *Foxa2* (Fig. 4D-I). All fragments showed the expected expression patterns: rostral node expressed only *Foxa2* and streak 1 expressed only *Fgf8*, whereas the border expressed both markers. Lateral pieces expressed primitive streak markers (Fig. 4J-M). A representative sample of six dissected pieces were dissociated and the cells counted. Each contained 100-230 cells, with an average of 149 cells.

Fragments from GFP transgenic embryos were grafted homotopically or heterotopically to stage-matched host embryos, which were then cultured for 48 hours. Embryos developed around 30-35 somites as expected, and all except one that received a graft contained labelled cells at the end of the experiment ( $n=84$ ). In 12/40 embryos in which streak was either the donor or host, small self-contained clumps were observed in addition to well-integrated stretches of cells in the axis, typically attached to the dorsal neural tube. They probably resulted from early expulsion of excess grafted tissue and, because they did not perturb axial development, they were not considered further. All grafted embryos were sectioned and their contribution to the axis and tail bud scored (see Materials and methods for the method of scoring). Section (i) below describes the fates of different regions of the primitive streak and node, and their commitment to these fates is detailed in sections (ii)-(iv).

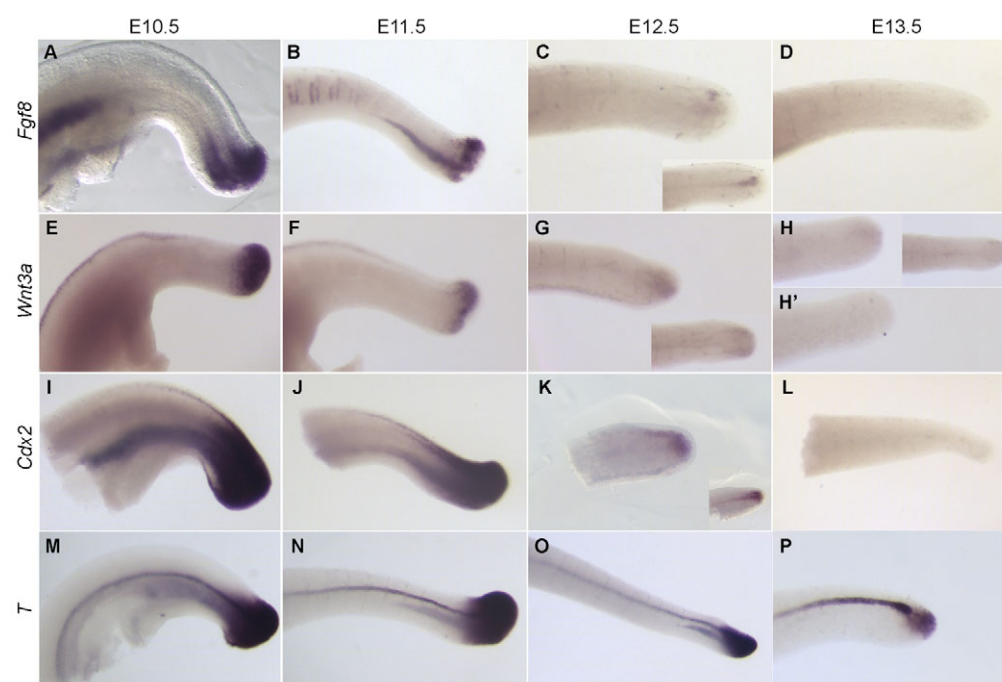
#### (i) Homotopic grafts of rostral node, border, streak and lateral regions contribute to distinct tissues and differentially colonise the CNH

After 48 hours whole-embryo culture, homotopic grafts had interspersed well with wild-type cells in the axis and tail bud (Fig. 5), and had populated different tissues in the axis. Grafted rostral node cells gave rise exclusively to notochord, and no labelled cells were found in the tail bud (Fig. 5A,Aa,Ab,Ac; Table 1). Border cells gave rise to ventral neural tube, somites and sporadic cells in the notochord. The notochord was, however, always colonised at its caudal end (Fig. 5B,Ba,Bb,Bc; Table 1). The contribution of border cells to somites was exclusively medial in the trunk but, in the tail, more lateral regions of the somite were colonised. In addition, the CNH and TBM (Fig. 5Bb,Bc) were colonised. Streak 1-4 grafts gave rise to somites and TBM, but not to CNH (9/10 embryos; Fig. 5C,Ca,Cb,Cc; Table 1). In these grafts, colonisation of the entire mediolateral extent of the somite was observed, even when the tissue originated from the caudal half of the streak. Whereas grafts of streak 1 were found exclusively in somites, streak 2-4 grafts





**Fig. 2. Gene expression in the primitive streak and tail bud.** Embryos were hybridised with in situ probes for *Fgf8* (A-B), *Evx1* (C-D), *Wnt3a* (E-F), *T* (G-H), *Foxa2* (I-J) or *Sox1* (M). (A,C,E,G,I) Whole-mount lateral views of E8.5 embryos; (A',C',E',G',I') corresponding sagittal sections. (B,D,F,H,J) E10.5 sagittal sections. Black and white arrows: end of the notochordal plate. Inset in G': transverse section of the caudal half of the node (at the level marked in G' by a white arrowhead), showing a cell in the ectodermal layer strongly expressing *T*. Black box in B: E10.5 chondroneural hinge (CNH). (K-L',N-O) Confocal images showing *Sox1-GFP* expression (green) and TO-PRO-3 counterstain (red). (K) Sagittal section of an E8.5 embryo. (L) Sagittal section of the tail tip of an E10.0 *Sox1-GFP* embryo. (L') Magnified view of the CNH region marked (white box) in L. (N,O) Transverse sections through the tail tip of an E10.0 embryo at the levels marked by vertical white lines in L (N, left line; O, right line). (N') Magnified view of the region marked (white box) in N, showing *Sox1-GFP*-positive cells in the notochord region (arrowhead). (P) Summary diagram. Blue, ectoderm expressing *Sox1* and *Foxa2*; brown, rostral prospective notochord expressing *T* and *Foxa2*; orange, caudal prospective notochord expressing *Fgf8*, *T* and *Foxa2*; dark purple, caudal node and streak ectoderm, and caudal ectoderm in the tail bud; light purple, streak mesoderm and TBM, both areas express *Wnt3a*, *Evx1*, *Fgf8*, *T* and *Sox1* (E10.5). Black boxes in P, E8.5 border and E10.5 CNH. not, notochord; nt, neural tube; hg, hindgut; np, neural plate; ps, primitive streak; tb, tail bud; tbm, tail bud mesoderm.

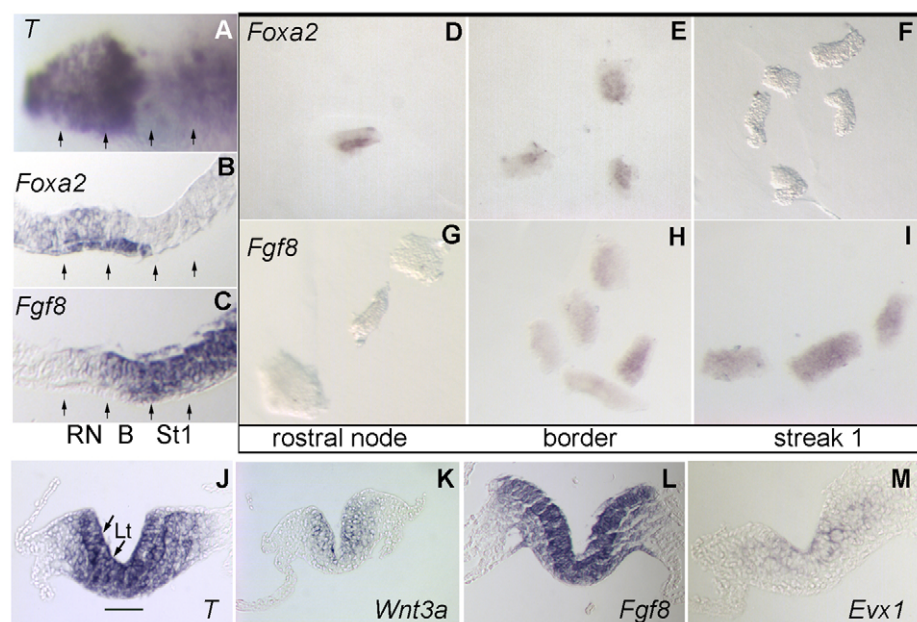


**Fig. 3. Decline in gene expression levels as tail elongation arrests.** Lateral views of tail buds hybridised with in situ probes for *Fgf8* (A-D), *Wnt3a* (E-H), *Cdx2* (I-L) or *T* (M-P). Insets in C, G, H, K show dorsal views. (H, H') Two E13.5 tails hybridised with *Wnt3a*, showing very low (H) or undetectable (H') expression.

additionally gave rise to intermediate mesoderm (Fig. 5Ca, arrowhead; Table 1). Streak 5 descendants contributed exclusively to intermediate and ventrolateral mesoderm (Fig. 5D, Da, Db, Dc; Table 1), and this included elongated cells that could be identified as endothelia at locations where they immediately abutted circulating blood (Fig. 5Da, inset). None of these grafts colonised the CNH. Therefore, among the cells at the midline, only descendants of the border are retained in the CNH.

Lateral border grafts colonised the neural tube and adjacent somites unilaterally, but did not contain any long-term progenitors, as judged by the absence of graft descendants from the tail bud ( $n=3$ ) (Fig. 5E, Ea, H; Table 1). Thus, all cells lateral to the border are en route for differentiation in the axis. Lateral grafts from regions located further

caudally showed a more complex pattern. Most (8/9) lateral 1-3-region grafts (Fig. 5F, Fa, Fb, Fc, H; Table 1) colonised a short stretch of 3-8 somites unilaterally on the grafted side (Fig. 5Fa, top), and thus were probably derived from pre-existing presomitic mesoderm included with the graft. Caudal to this stretch, the neural tube was colonised predominantly on the grafted side (Fig. 5Fa, bottom). Unlike border descendants, which colonised the ventral third of the neural tube, lateral 1-3-region descendants tended to integrate more dorsally, although a minority of descendants were found in the ventral midline (Fig. 5Fa, bottom). At the same axial level as the neural tube contribution in most (6/8) embryos, grafted cells also integrated bilaterally in somites. Together, these data indicate that some cells from lateral 1-3 grafts encroach on the midline and produce mesoderm



**Fig. 4. Dissection of pieces for grafting.** (A) Ventral view of rostral node (RN), border (B) and streak 1 (St1) regions of an E8.5 embryo hybridised with a *T* (brachyury) in situ probe; (B, C) sagittal sections showing the same region in embryos hybridised with *Foxa2* (B) or *Fgf8* (C). Arrows delimit the regions dissected. (D-I) In situ hybridisation of dissected pieces. The rostral node expresses *Foxa2* (D), but not *Fgf8* (G); streak 1 expresses *Fgf8* (I), but not *Foxa2* (F); however, the border expresses both markers (E, H). (J-M) Transverse sections through mid-streak (St2) of E8.5 embryos hybridised with *T* (J), *Wnt3a* (K), *Fgf8* (L) and *Evx1* (M). Line in J marks the midline, arrows mark the lateral region grafted (Lt).



Table 1. Contribution of cells grafted homotopically

Graft site	No. incorp./no. grafted	Transient unilateral						Unilateral and/or bilateral, continuous with the tail bud										Tail bud			Ectopic structure
		Rostral limit	Neural tube	Noto-chord	Paraxial mesoderm	Intermediate mesoderm	Vento-lateral mesoderm	Rostral limit	Neural tube	Noto-chord	Paraxial mesoderm	Intermediate mesoderm	Vento-lateral mesoderm	CNH	TBM						
Midline	Rostral node	5/5						18.0±3.5 <sup>§</sup>	0	5	0	0	0	0	0	1 <sup>†</sup>	0	0			
	Border	8/8						12.8±2.4	8	7 (5 low)	8	0	0	0	8	8	0	0			
	Streak 1	6/6						13.2±1.6	1* (low)	0	6	0	0	0	1*	6	1*	0			
	Streak 2-4	4/4						14.0±2.3	0	0	4	4	0	0	0	4	0	0			
	Streak 5	2/2						22.5±0.7	0	0	0	0	2 (1 low)	2	0	2	0	0			
Lateral	Border	3/3	7.7±0.6	3	0	1	0	0													
	1-3	9/9	13.1±1.5	0	0	9	9	0	8	0	7	7	0	8	8	1*	0	0			
	4	1/1	11	0	0	1	1	21	1	0	1	1 (low)	1 (low)	1	1	0	0	0			
	5'	2/2						21.5±2.1 <sup>§</sup>	0	0	0	2	2	0	2	0	0	0			

The number of grafted embryos with incorporated cells in each tissue scored is shown. Shading indicates that >50% of the embryos had incorporated cells in the tissue scored.

\*One embryo in this batch contained an ectopic patch of cells that joined the neural tube at the posterior end continuous with the CNH.

†Grafted cells integrated unilaterally in both embryos.

§Very few cells (<5).

§A clump of partially incorporated cells was also found anteriorly at somite 8.4±2.6 in five embryos, close to the presumed graft site.

\*A small number of endothelial cells extend more anteriorly as far as somite 15±2.8.

No. incorp./no. grafted, total number of embryos containing incorporated cells/total embryos receiving a graft; CNH, chordoneural hinge; TBM, tail bud mesoderm.

Table 2. Contribution of cells grafted heterotopically

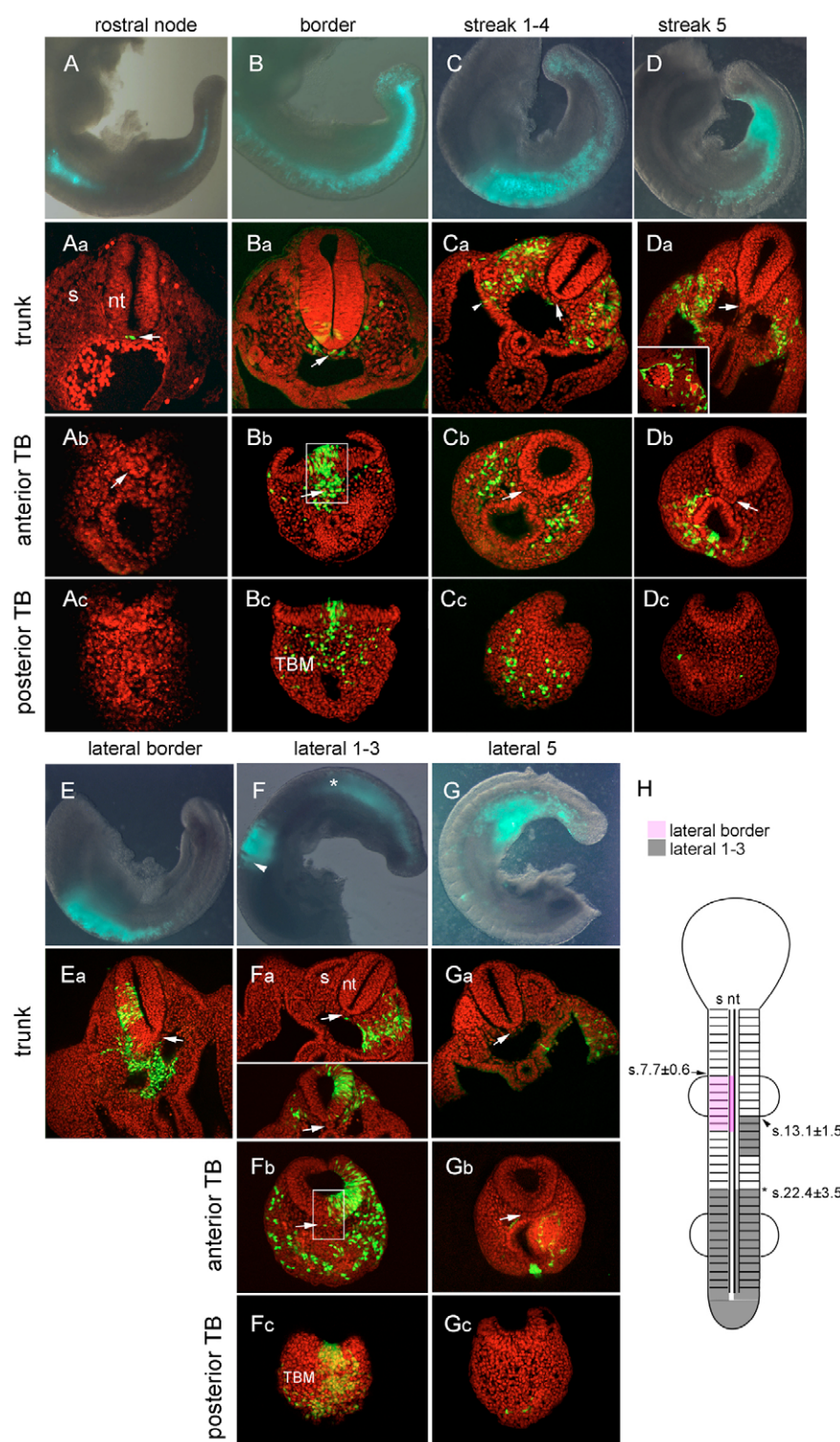
Donor	Graft site	No. incorp./no. grafted	Rostral limit	Axis				Tail bud		Ectopic structure
				Neural tube	Notochord	Paraxial mesoderm	CNH	TBM		
Border	Rostral node	7/7	16.2±4.2*	4 (2 low)	6	4 (3 low)	5 <sup>†</sup>	1	1	
	Streak 1	7/7	13.6±1.1	6 (4 low)	4 (low)	7	7	7	2	
Rostral node	Border	7/7	16.1±3.9	7	7 (low, TB)	1 (low)	7	3 <sup>†</sup>	4	
	Streak 1	7/7	18.4±7.7	5	6 (5 low)	3 (low)	7	4	6	
Streak 1	Border	7/7	12.4±1.9	4 (TB, 2 low)	0	7	6	7	1	
	Rostral node	5/9	12.4±1.9	3 (2 low)	4 (2 low, TB)	4 (1 low)	3	3	5	

\*A clump of partially incorporated cells is also found anteriorly at somite 8.5±1.0 in four embryos, close to the presumed graft site.

†In notochord end only.

§Very few cells (<5).

Abbreviations and shading: see Table 1. TB, tail bud.



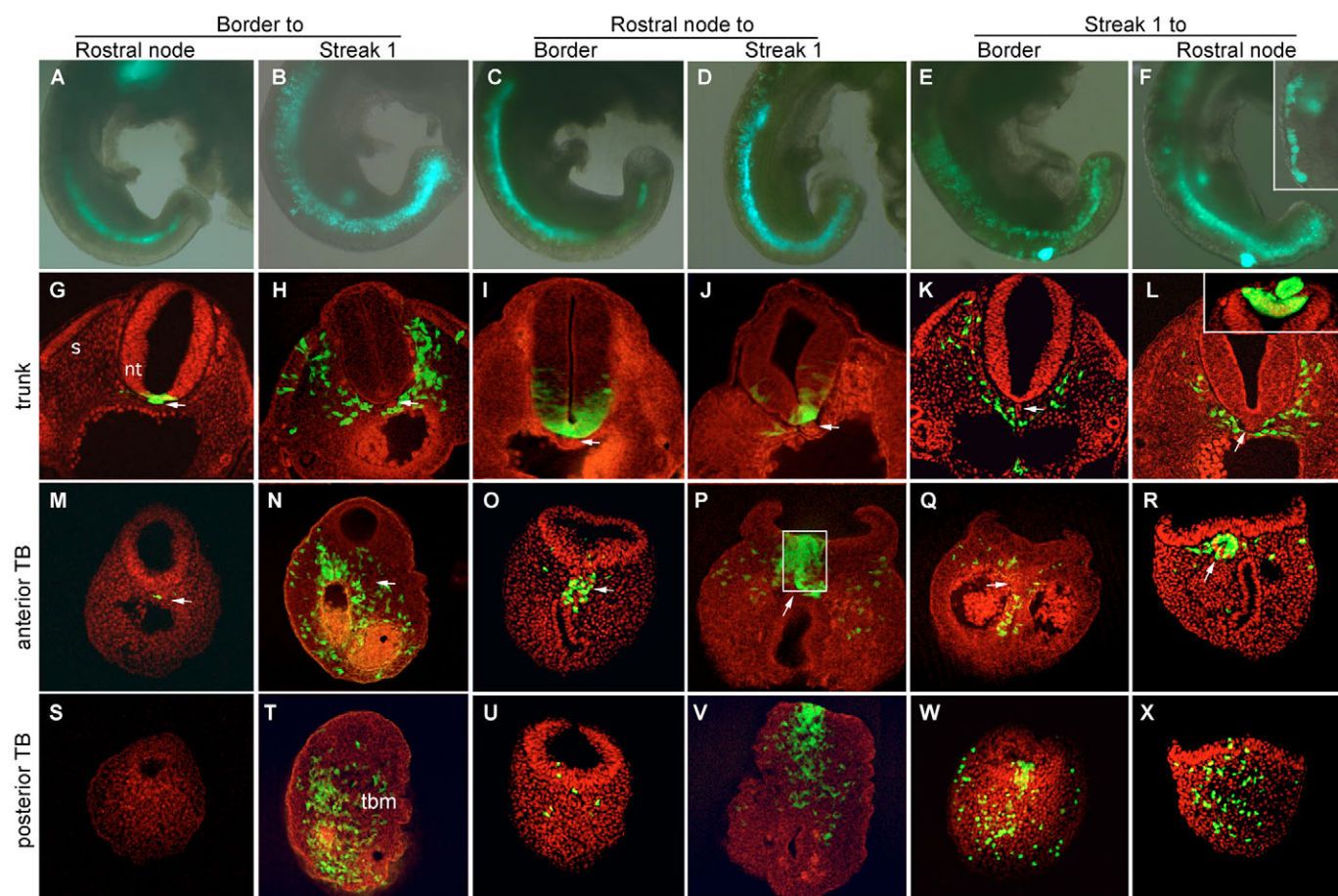
**Fig. 5. Homotopic grafts.** (A-G) Trunk and tail of embryos showing incorporation of GFP-labelled cells in homotopic grafts, as indicated. Transverse confocal sections through representative embryos are shown in panels labelled with a lowercase letter. Sections Aa...Ga, mid trunk; Ab...Gb, rostral tail bud (chordoneural hinge, CNH); Ac...Gc, the bud caudal to the notochord end. (Ca, arrowhead) Labelling in intermediate mesoderm. (Da, inset) Transverse confocal section through a different embryo showing endothelial contribution of labelled cells. (Fa) Top section, rostral-most unilateral contribution in the somites in lateral 1-3 grafts (arrowhead in F and H); bottom section, caudal unilateral neural tube and bilateral somite contribution (asterisk in F and H). White arrows, notochord; white boxes in Bb and Fb, CNH. (H) Diagram showing contribution of the lateral border and lateral 1-3 regions to the somites and neural tube. The rostral-most somite labelled is represented as  $\text{average} \pm \text{standard deviation}$ . s, somite; nt, neural tube; TB, tail bud; TBM, tail bud mesoderm.

(Fig. 5Fa, bottom). Cells from these grafts also contributed to the medial part of the neur ectoderm dorsal to the CNH and, to a lesser extent, to the CNH itself (Fig. 5Fb,Fc; Table 1).

Lateral 4-5 grafts gave rise to lateral mesoderm and to cells associated with blood vessels (Fig. 5Gb). The single lateral 4 graft also produced labelled CNH cells, as well as neural tube and some bilateral somitic mesoderm (data not shown), whereas lateral 5 grafts contributed unilaterally and showed no labelled cells in either the

trunk neur ectoderm or in the CNH (Fig. 5G,Ga,Gb,Gc; Table 1). Thus, the lateral 4 position might span a transition zone between neural/somite and ventrolateral mesoderm progenitors.

We examined the rostral limit of labelling in each embryo, using the somite level as a morphological landmark. In general, a more rostral position in the streak correlated with a more rostral limit of labelling (Table 1) and corresponded well to that seen when labelling the equivalent regions in situ using *DiI* (Wilson and



**Fig. 6. Heterotopic grafts.** (A-F) Trunk and tails of embryos receiving a heterotopic graft as indicated. (G-X) Transverse confocal sections through a representative embryo. Sections through body and tail are at the same levels as in Fig. 5. Insets in F and L, embryos in which donor cells did not incorporate well in the axis and tail bud but formed an ectopic structure. Arrows indicate the notochord. White box in P, chordoneural hinge (CNH). s, somite; nt, neural tube; tbm, tail bud mesoderm.

Beddington, 1996). Thus, grafted cells generally incorporate quickly in the host embryo. One exception was that rostral node integration in notochord occurred around the level of somite 18, suggesting there was a delay in integration of this tissue. The rostral limit of the short unilateral somite stretch derived from lateral 1-3 grafts (somite 13) corresponded almost exactly to that from the adjacent midline streak 1-4 grafts (somite 13-14), even though, as a more differentiated derivative of streak, it would be expected to lie more rostrally. This implies that some cells exit rapidly from the midline to the adjacent presomitic mesoderm. The rostral limit of the predominantly neural lateral border derivatives was at somite 7-8 (Fig. 5H, Table 1) and the total labelled region was no more than 9 somite lengths. Neural cells derived from lateral 1-4 grafts stretched from the somite 22 level caudally, although this anterior limit was variable (range: somite 15-27). The lack of overlap between lateral border and lateral 1-4 derivatives suggests that there is little mixing between long-term neural progenitors and their more differentiated progeny located further rostrally. Finally, streak 5 and lateral 5 grafts colonised much more caudal regions (around the level of somite 21-22) than streak 1-4 and lateral 1-4 grafts (around somite 13). Because somite 20 is derived from progenitors in the streak at E9.5, it appears that the lateral mesoderm is delayed in its exit from the streak or lateral regions by about 24 hours relative to the presomitic mesoderm.

In summary, the bulk of cells at the caudal midline of the E8.5 embryo are destined for mesoderm, with rostral positions generally colonising medial regions of the axis. In the midline, only the border gives rise to both neural tube and mesoderm. Lateral to the streak, neural and somite progenitors seem to overlap extensively in the ectoderm layer. In our search for streak regions that are retained in the CNH, we found that the majority of the CNH is derived from the border, with a minor contribution from lateral regions.

## (ii) The border can produce rostral node and rostral streak derivatives

The border, although it integrated in all dorsal axial derivatives, did not contribute extensively to trunk notochord or to lateral regions of the trunk somites. To test whether this was because descendants were already committed to form medial somites and posterior notochord, the border was transplanted to the rostral node or streak 1 position. In the rostral node, border cells formed large stretches of trunk notochord (Fig. 6G,M, Table 2), whereas, in the streak 1 position, they contributed much more prominently to the whole somite (Fig. 6H,N,T, Table 2). This implies that it is the position of border cells, and not their intrinsic differentiation capacity, that directs their low contribution to notochord and lateral regions of the trunk somites in situ. However, in both sets of grafts, there was a consistent low-level contribution to tissues that were not colonised



in node or streak homotopic grafts, including the CNH, suggesting that the border retained some of its original characteristics in these ectopic environments (Table 2). Thus, border cells can change fate in response to node or streak environments, although they form a broader spectrum of differentiated cell types than node or streak cells *in situ*. Notably, border descendants were retained in the CNH in these heterotopic grafts.

### (iii) Rostral node fragments can generate extensive ventral neural tissue

To determine whether the rostral node was committed to forming trunk and anterior tail notochord, this tissue was transplanted to the border or to the streak 1 position. When challenged with the border environment, rostral node descendants lost virtually all of their contribution to notochord except for a small stretch in the tail bud, a pattern reminiscent of border cells *in situ* (Fig. 6O, Table 2). Instead, rostral node gave rise almost exclusively to ventral neural tissue in the axis (Fig. 6I, Table 2). The CNH was also colonised (Fig. 6O, Table 2), indicating that rostral node cells can act as neural progenitors when given appropriate conditions. Furthermore, rostral node cells grafted to streak 1 (which does not itself contain neural progenitors) also generated extensive neural tissue and the CNH (Fig. 6J,P,V; Table 2). Only 3/7 grafted embryos showed any paraxial mesoderm colonisation (Table 2), which was minimal in all three embryos (data not shown). Therefore, the rostral node contains cells that can act as neural progenitors, although these cells do not show this property *in situ*.

### (iv) The rostral primitive streak has a limited differentiation capacity

Whereas primitive streak cells *in situ* populated the entire mediolateral extent of the somite, streak 1 cells grafted to the border environment tended to colonise medial positions in a pattern characteristic of border homotopic grafts (Fig. 6K). However, they did not generate notochord, and only a very small number of cells was present in caudal ventral neural tube and in the CNH (Fig. 6W). In striking contrast to all other graft types, streak 1 cells grafted to the node showed a very poor level of incorporation (Table 2). In a high proportion of embryos, these unincorporated cells also formed ectopic neural-like structures that joined and separated from the endogenous neural tube along the axis (Fig. 6F,L, inset). Of the cells that incorporated well in the axis, most formed medial paraxial mesoderm (Fig. 6L), although some examples of apparently bona fide incorporation in the other axial tissues were present. In the five embryos that showed incorporation at some level along the axis, three colonised the CNH in the tail bud (Fig. 6R, Table 2). In summary, the streak shows the least plasticity on ectopic grafting, but instead tends to form ectopic neural-like structures. However, cells were sometimes present in the CNH after culture. Retention in the CNH is thus a property of all ectopic grafts.

## DISCUSSION

Using careful gene expression mapping, we have established a continuity of tissue topology between the primitive streak and tail bud. This has identified the node-streak border as the earlier equivalent of the CNH. We have then shown, using homotopic grafts, that cells in the border form the majority of CNH progenitors, despite also producing extensive differentiated tissues in neurectoderm, notochord and somites. A secondary contributor to the CNH is found in the lateral ectoderm, where progenitors of caudal neurectoderm and mesoderm are located.

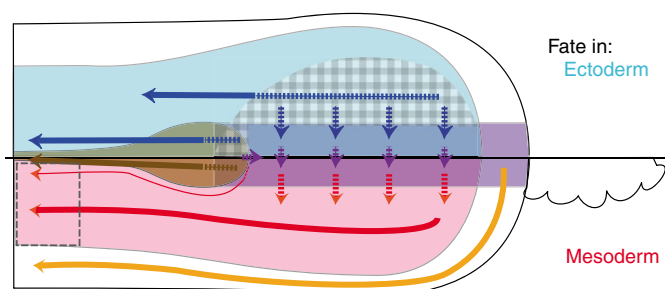
Finally, we have shown that border cells are highly adaptable to ectopic transfer to new environments, whereas rostral node and streak are more limited in their differentiation options.

### Gene expression in the streak and tail bud

Our expression study showed that E8.5 streak and node expression domains and their relative orientation are preserved in the CNH, highlighting a similarity in the general layout of the streak and tail bud, and particularly between the border and CNH (Fig. 2P). This similarity in gene expression is reflected in the unique ability of these two regions to give rise to notochord, somites and ventral neural tube (Cambray and Wilson, 2002) (this study). In later axis elongation, the levels of gene expression decline (Fig. 3 and see Fig. 1 in the supplementary material). *Cdx2* and *Wnt3a* are essential for complete axis elongation, because mutants lacking wild-type levels of these genes have short tails (Takada et al., 1994; Chawengsaksophak et al., 2004). Despite this requirement, *Wnt3a* expression decreases between E10.5 and E11.5, with the progenitors of a further 20–25 somites still present. Its expression is almost absent in the progenitors of the last 10 somites, which also express very little *Cdx2*. *Fgf8* levels follow a similar schedule to *Wnt3a*. *Fgf8* has not been shown to be essential for this phase of axis elongation because null mutations terminate at the gastrulation stages (Meyers et al., 1998), but FGF signalling is essential for this process (Partanen et al., 1998). Therefore, it is possible that lowered levels of *Fgf*, *Wnt* and *Cdx2* transcripts lead to the termination of axis elongation after a delay, perhaps due to a slow decline in FGF/Wnt signalling levels. If this is true, the supply of axial progenitors might be exhausted by E13.5 as a direct consequence of an earlier loss of essential components needed for maintenance of the progenitors. However, axial progenitor capacity in E12.5 and E13.5 tail bud cells can recover when grafted to younger embryos (Cambray and Wilson, 2002; Tam and Tan, 1992), and so these cells must remain responsive to signals from their environment, presumably including FGF/Wnt signalling from neighbouring cells.

### A new fate map for the late mouse primitive streak and surrounds

The fate map that we have deduced from homotopic grafting at E8.5 (Fig. 7) confirms and extends that of Wilson and Beddington (Wilson and Beddington, 1996) and is similar in essence to that of other vertebrates, in particular, the chick (Psychoyos and Stern, 1996). However, our results reveal a number of interesting differences between the mouse and chick. In chick, at equivalent somite stages [Hamburger and Hamilton (HH)7–9], although the rostrocaudal arrangement of progenitors is the same as that in mouse, the proportion of the streak fated for the different mesoderm types differs. Somites are produced from a much higher proportion of the mouse streak than the chick (approximately 80% versus 20% of streak length). In the chick, the caudal 50% of the streak length contains extraembryonic mesoderm progenitors, whereas, in mouse, all extraembryonic mesoderm has exited the streak by the early headfold stage. Lateral mesoderm is produced between approximately 50–80% of the streak length in the chick (where 90–100% is the node), whereas, in mouse, we found that lateral mesoderm progenitors are confined to the caudal 20% of its length. In this respect, the fate map of chick at 7 somites resembles the fate map of the mouse E7.5 streak (late streak, equivalent to approximately stage 4+ in chick) (Smith et al., 1994). In agreement with data in chick, we found that cells in or very close to the node exclusively produce the medial part of the somite in the trunk (Selleck and Stern, 1991; Freitas et al., 2001). However, fate maps of the primitive streak in chick suggest that descendants of the rostral



**Fig. 7. Cell fate and movements at the caudal end of the E8.5 embryo.** Tissues are colour-coded as follows: blue, neurectoderm (flattened so that the dorsal neural tube lies laterally); brown, notochord; pink, paraxial mesoderm; white fill and orange arrow, lateral mesoderm; purple, primitive streak. The checked area contains progenitors of chondroneural hinge (CNH) cells and neural progenitors retained in the tail bud at E10.5. Notice the region overlap. Arrows represent the movement of cells and are colour-coded as follows: blue, neurectoderm; brown, axial mesoderm; red, paraxial mesoderm; orange, lateral mesoderm. Broken arrows, movements of progenitor cells inside the progenitor zone. As well as a net movement rostrally to neural tube from the progenitor zone, lateral progenitors can move towards the midline (blue broken arrows), form mesoderm (purple arrows) and move towards presomitic mesoderm (red arrows). Cells at the midline exit to presomitic mesoderm, and might join a pre-existing stream of presomitic mesoderm. In the border (junction of checked, purple and brown regions), cells exit to ventral neurectoderm, notochord and medial somites (thin red arrow). They might also exit caudally to the primitive streak (purple arrow). Box enclosed by a broken line indicates the most recently formed somite.

streak behind the node are also at least partially restricted to the medial somite, whereas regions located further caudally contain at least some laterally restricted progenitors (Psychoyos and Stern, 1996). In our experiments, cells abutting the border (streak 1) generally produced the entire somite, whereas some streak 4-derived cells extended into medial regions, although there was a tendency for a more lateral somite contribution in more caudal streak grafts. The overlap of streak-derived cells with the domain populated by border descendants raises the possibility that the somite-organising capacity of medial paraxial mesoderm (Freitas et al., 2001) is initiated by a subpopulation of medial progenitors, rather than the entire domain.

Fate mapping experiments have previously shown that ectoderm-to-mesoderm transition continues late into axis elongation (Cambray and Wilson, 2002; Wilson and Beddington, 1996), generating cells that will pass through the presomitic mesoderm to the somites. Although it is known that axial progenitors at E8.5 reside in or close to the streak (Snow, 1981), it is not known how close to the midline these progenitors are. We have shown that there is significant movement from lateral ectoderm to the midline at this stage, consistent with results in the chick (Imura and Pourquié, 2006). Meanwhile, streak cells exit completely from the midline ectoderm, so that cells from the border and lateral ectoderm are the only occupants of the E10.5 CNH. Within the mesoderm, some cells exit quickly from the midline to presomitic mesoderm, as judged by the identical rostral limits of streak 1-4 and lateral 1-4 grafts in mesoderm. However, because some streak-derived cells at E8.5 are found in the TBM 48 hours later, the period of exit from midline mesoderm to presomitic mesoderm is relatively prolonged.

### Commitment of border cells to their fates

The border contains both neural and somite progenitors. These are most probably located in the ectoderm layer, because labelling the ventral layer of the node (including the cells in the ventral part of the border) shows no neural or somitic descendants (Wilson and Beddington, 1996; Cambray and Wilson, 2002). However, the border additionally contains notochord progenitors. Labelling the ectoderm of the chick node (Selleck and Stern, 1991) shows that cells from this layer can colonise notochord. Therefore, at least some of the material for the mouse notochord might be derived from the ectoderm. Interestingly, in the border ectoderm, a few cells express *T* very highly (Fig. 2G' and inset). Because ectoderm cells in the node can produce notochord (and not vice versa), these ectoderm cells that strongly express notochord markers might therefore be in transit to the notochord. The apparent continuity between *Sox1*-positive cells in the CNH ectoderm and the notochord (Fig. 2N,N') suggests that, here too, cells marked by *Sox1* continue to exit the CNH ectoderm to notochord.

It is interesting that progenitors of notochord, neural tube and somites are contained in this small region. Selleck and Stern (Selleck and Stern, 1991) showed that single cells in the chick node can produce more than one tissue type: namely, notochord and neural tissue or notochord and somites. Because the ectoderm layer can produce all three tissues, the border ectoderm could comprise a population of multi-fated or multipotent cells, although a mixture of cells each fated for one tissue type cannot be ruled out from our experiments. On ectopic grafting, border cells can either produce predominantly somites or notochord, depending on their location (Fig. 6). These ectopic grafts show few unincorporated cells, suggesting that most cells of the graft could participate in the development of either somites or notochord. The results of these ectopic grafting experiments thus imply that at least some border cells can change fate in response to environmental cues and therefore might be multipotent. A similar study on earlier-stage chick embryos (Selleck and Stern, 1992b) also shows that a region of the node containing both notochord and somite precursors (the lateral sector) can adapt to heterotopic grafting to regions of prospective notochord (medial sector) or somites (streak) by producing more or less somitic mesoderm.

### Commitment of rostral node and streak 1 cells to their fates

Heterotopic grafts of rostral node and streak 1 showed that they are less potent than the border, because grafting them ectopically did not confer the ability to produce the full range of border-derived tissues. Indeed, heterotopic streak 1 grafts to the node showed a reduced level of graft incorporation and, in the embryos that maintained the graft, many of the resulting cells formed ectopic structures, showing that streak cells are not readily adaptable to the node environment (Fig. 6). However, rostral node cells, which contain no neural progenitors in situ, were capable of converting to neural progenitors on heterotopic grafting. Most strikingly, there was significant neural contribution when rostral node was grafted to streak 1 (Fig. 6J,P). Because neither donor nor host contained any neural progenitors, the streak 1 environment unveiled a neural progenitor capacity inherent in (but not exhibited by) the rostral node ectoderm. It is likely that the node environment represses neural progenitor identity, because border cells grafted to the node showed a reduced neural fate compared with border homotopic grafts, and also produced only notochord and not ectoderm in the CNH (Table 2). Interestingly, heterotopic grafts of node and streak all contributed to the CNH. This might result from a delay in the integration of an ectopic piece

of tissue, leading to delayed exit from the progenitor region. Because the rostral limit of grafted cell contribution is generally slightly more caudal than that of the corresponding homotopic graft (Fig. 5H, Table 2), this might be the case. However, it is also possible that, in some cases, the ectopic tissue combination imposes progenitor status upon grafted cells that would otherwise have been en route for axial differentiation.

The latter possibility is consistent with the results of our previous study (Cambray and Wilson, 2002), because some node grafts that produced no somites and thus probably excluded the border region could be retransplanted once, giving rise to both axis and CNH. These must therefore have re-acquired at least some aspects of progenitor status, and suggests that there might be some plasticity in the rostral node and rostral streak populations. The normal encroachment of lateral ectoderm cells on the CNH would then be consistent with an ability of cells to acquire long-term progenitor status based on their position, and not on an inherent property of the cells. Joubin and Stern (Joubin and Stern, 1999) showed that the early chick node normally receives incoming lateral cells up to stage 3+ and, therefore, until this stage, the node consists of a transient population defined by molecular interactions. However at later stages, they observed little movement of lateral cells towards the node. Our study shows that some cells still move towards the border descendant after the mouse equivalent of stage 4 (mid-late streak, E7.5) but from more caudal positions. Therefore, the imposition of progenitor status by the border and CNH might be a continuous process throughout axis elongation.

### Interchangeability of neurectoderm and somite progenitors

Neural progenitors in the lateral ectoderm are closely associated with mesoderm progenitors, because lateral grafts give rise to neurectoderm and somites at similar ( $\leq 3$  somites distant) axial levels (Fig. 5Fa,Fb, inset; and data not shown). Three embryos receiving a graft in the lateral 1-3 position were particularly informative. In two of these, no somitic contribution was evident, and the third showed no neural contribution. This suggests that, although neural and somitic progenitors overlap in the lateral ectoderm, they do not exactly coincide. It is therefore possible that position in the ectoderm determines a neural or somitic fate. However, we could not distinguish neural and somitic progenitors on the basis of the gene expression markers used, suggesting they are related cell types.

Apparent interchangeability of neural and somitic progenitors was seen in embryos lacking *Wnt3a*, its target (brachyury; *T*), or *Tbx6*. Here, cells normally destined for mesoderm differentiated inappropriately as neural tissue (Yoshikawa et al., 1997; Yamaguchi et al., 1999; Chapman and Papaioannou, 1998). Signalling via *Fgfr1* is also required for *Wnt3a*-mediated mesoderm differentiation and *Tbx6* expression (Ciruna and Rossant, 2001). Furthermore, ectopic expression of *T*, *Fgf* and *Wnt* family members in *Xenopus* can drive mesoderm formation in naïve ectoderm (Cunliffe and Smith, 1992; Slack et al., 1990; Sokol, 1993). Thus, activation of this cascade in prospective mesoderm appears sufficient to divert cells from a neural to mesodermal lineage, suggesting that the two progenitor types have a related differentiation capacity.

### Resident cells and stem cells

Clonal analysis in the chick has indicated a stem cell population for somites and notochord in the node (Selleck and Stern, 1992a). In the mouse, retrospective clonal lineage analyses also point to a stem cell progenitor of the myotome (Nicolas et al., 1996) and postcranial

spinal cord (Mathis and Nicolas, 2000). The primitive streak and tail bud are the only known locations for axial progenitors during axial elongation. Therefore, these putative stem cells would have been located there at some point in their history, although this was not directly shown because only differentiated cells, and not the progenitors, were scored. Can these myotome and spinal cord stem cells be linked with the border?

The E8.5 border is the major contributor to the CNH, the only region of the tail bud that later shows stem cell-like properties (Cambray and Wilson, 2002). Therefore, the border, as the progenitor of a candidate stem cell-containing region, might itself contain stem cells. In this context, it is interesting that clonal prospective fate mapping a day earlier, at E7.5, has identified apparently resident cells in a small region at the caudal end of the node, apparently corresponding to the border (Forlani et al., 2003). Indeed, early in gastrulation (E6.5), the incipient node is the only region to contain apparently resident cells (Lawson et al., 1991). Therefore, prospective fate mapping studies consistently point to the node region (and when it appears, the border), as a site for resident cells throughout axis elongation. Although residence in the progenitor region does not by itself demonstrate that cells are axial progenitors or stem cells, it is a prerequisite for such cells. Thus, although each study only deals with a short period in axis elongation, stem cells similar to those proposed by Selleck and Stern (Selleck and Stern, 1992a) in the chick might be present from the beginning to end of axis elongation, localised successively in the incipient node, border and CNH region of the mouse.

The above lineage studies, together with our previous serial transplantation of CNH cells, suggest that axial progenitors can behave as stem cells. However, our data show that these progenitors undergo changes in gene expression and, therefore, they are not strictly self-renewing *in vivo*, but are maturing. Thus, experimental perturbation might enable self-renewal of progenitors that would normally evolve progressively.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/15/2829/DC1>

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