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The generation of vertebral segmental patterning in the chick embryo

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Abstract

We have carried out a series of experimental manipulations in the chick embryo to assess whether the notochord, neural tube and spinal nerves influence segmental patterning of the vertebral column. Using *Pax1* expression in the somite-derived sclerotomes as a marker for segmentation of the developing intervertebral disc, our results exclude such an influence. In contrast to certain teleost species, where the notochord has been shown to generate segmentation of the vertebral bodies (chordacentra), these experiments indicate that segmental patterning of the avian vertebral column arises autonomously in the somite mesoderm. We suggest that in amniotes, the subdivision of each sclerotome into non-miscible anterior and posterior halves plays a critical role in establishing vertebral segmentation, and in maintaining left/right alignment of the developing vertebral elements at the body midline.

Key words: annulus fibrosus; intervertebral disc; notochord; nucleus pulposus; *Pax1*; sclerotome; segmentation; somite; vertebral column.

Introduction

The segmented repeat pattern of the vertebral column clearly reflects its developmental origins from the embryonic somites. Since Robert Remak's pioneering observations (Remak, 1855), it has been widely accepted that vertebral segmentation in birds and mammals involves a process of somite 'resegmentation' (neugliederung) along the long axis. Remak pointed out that while vertebrae and axial musculature derive segmentally from their respective somite components, the sclerotome and myotome, there is a half-segment shift in the alignment between them during development. As a result, the axial muscles can make intervertebral attachments, promoting motility of the vertebral column and body trunk. Remak also detected an anatomical subdivision of the sclerotomes into two halves, anterior (cranial) and posterior (caudal), and accounted for the shift by proposing that each vertebral body forms by a recombination of the adjacent half-sclerotomes of neighbouring somites on each side of the midline, while the myotomes retain their original segmental positions. The subdivision of the sclerotome also imposes segmentation on the outgrowing spinal nerves (Keynes & Stern, 1984; Kuan et al. 2004), and creates alternating, non-miscible populations of cells in consecutive sclerotomes along the long axis (Stern & Keynes, 1987).

Several morphological studies have disputed the existence of sclerotome resegmentation, based largely on a lack of visible segmentation in the initial cell population that forms the axial peri-notochordal sclerotome, where resegmentation is held to take place (for review, see Verbout, 1976). The evidence of such exclusively descriptive studies is not conclusive, however, and the majority of more recent lineage studies have been generally consistent with resegmentation. Thus, single somite transplants from quail to chick embryos have shown that one sclerotome contributes cells to the adjacent halves of neighbouring vertebral bodies, and to the annulus fibrosus of the intervertebral disc between them. Correspondingly, a single vertebral body, its neural arch and attached processes are derived from two adjacent half-sclerotomes (Bagnall et al. 1988; Bagnall & Sanders, 1989; Ewan & Everett, 1992; Huang et al. 1996; Aoyama & Asamoto, 2000). Resegmentation has also been confirmed by Huang et al. (2000), who took the extra step of ensuring correct orientation of transplanted somites by grafting one and a half somites from quail to chick embryos. Other quail–chick transplant studies have shown that particular sub-regions of the sclerotome give rise to individual vertebral components, for example the pedicle of the neural arch from posterior half-sclerotome (Goldstein & Kalcheim, 1992), and the annulus fibrosus and rib from the 'somitocoele' cells that colonize the

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cavity of the early epithelial somite (Huang et al. 1994; Christ et al. 2007).

A resegmentation process therefore seems likely to generate the segmented pattern of the mature vertebral column, and the question arises whether additional signals from tissues extrinsic to the sclerotome are also involved in creating or maintaining the segmental vertebral pattern. Studies in both chick (Stern & Keynes, 1987) and zebrafish (Morin-Kensicki et al. 2002) have shown that cells from one half-sclerotome can contribute to two adjacent vertebrae, rather than just one as resegmentation predicts. Moreover, although vertebral segmentation may be sclerotome-dependent in the medaka (Inohaya et al. 2007; Spoorendonk et al. 2008), where it is also dependent on *wnt4b* production by the floor plate (Inohaya et al. 2010), recent studies of vertebral segmentation in two other teleost species, the zebrafish (*Danio rerio*; Fleming et al. 2001, 2004) and Atlantic salmon (*Salmo salar*; Grotmol et al. 2003, 2005), have suggested that osteogenic activity in the notochord generates the part of the vertebral body (chordacentrum) that immediately surrounds it. This raises the possibility that segmentation of amniote intervertebral discs and intervening centra may likewise be influenced by segmental signalling from the notochord, consistent with the well-documented persistence of notochord cells that form the nucleus pulposus of the intervertebral disc (Balfour, 1881; Peacock, 1951; Walmsley, 1953; Urban et al. 2000; Choi et al. 2008). A further consideration is that, according to the resegmentation hypothesis, the left and right halves of the perinotochordal sclerotome develop independently of each other while simultaneously maintaining precise segmental alignment at the axial midline. An additional midline-derived mechanism might therefore be anticipated, ensuring that left/right registration of the mesenchymal sclerotomes is preserved as their cells migrate to surround the notochord and the neural tube, a lengthy process that may occupy several days during amniote development.

Accordingly we have undertaken a series of manipulation and transplant experiments in the chick embryo to assess whether the notochord, neural tube and developing spinal nerve components influence segmental vertebral patterning. Using perinotochordal expression of *Pax1* in the developing sclerotome as a marker for segmentation of the developing intervertebral disc, our results exclude such a mechanism. They indicate that segmental patterning of the amniote vertebral column, in contrast to the teleost species noted above, derives autonomously in the somite mesoderm from persisting midline registration of the bilateral sclerotomes during vertebral development. We argue that the subdivision of each sclerotome into non-miscible, and hence non-motile, anterior and posterior halves is critical in establishing vertebral segmentation and in maintaining left/right registration of the ventral vertebral elements as they merge at the axial midline.

Materials and methods

Embryo manipulations

Fertilized hens' eggs (Winter Egg Farm, Hertfordshire, UK) were incubated at 38 °C to obtain embryos at stage 11–14 (Hamburger & Hamilton, 1951). Host eggs were windowed, and 5–100 μL of a 1 : 10 mixture of India ink (Fount India, Pelikan) and phosphate-buffered saline (PBS) was injected into the subblastodermal space. The window was then lined with silicone grease, allowing the embryo to be floated above the surface of the egg within $\sim$ 1 mL of calcium/magnesium-free Tyrode's solution (CMF), the latter having being introduced into the window using a pipette.

For notochordectomy and neural tube removal (neuralectomy), a microscalpel (John Weiss, Milton Keynes, UK) was used to cut the vitelline membrane and deflect it away from the operated region. Longitudinal cuts were made between the neural tube/notochord and somite mesoderm on either side, extending 6–9 somite-lengths and including 4–9 somite-lengths of notochord and neural tube at the level of the pre-somite mesoderm (PSM). A single transverse cut was then made across both neural tube and notochord at the level of the anterior end of the longitudinal cut, allowing the neural tube and notochord to be cleaved easily from the underlying endoderm as a combined unit. Removal was completed by a second transverse cut through the neural tube and notochord at the posterior end of the longitudinal cut. The cleavage plane lay between the notochord sheath and the underlying endoderm, so notochord removal left the endoderm intact with no passage of ink across it. As the notochord was removed together with its sheath, there were no residual notochord cells (Fig. 2).

For PSM reversal, a longitudinal cut was made between the neural tube/notochord and PSM on one side, and between the PSM and intermediate mesoderm on the same side of the embryo. Both cuts extended posterior from the second most recently-formed somite for 8–10 somite-lengths. Transverse cuts were made at the anterior and posterior ends of these cuts, and the PSM removed using the microscalpel. This was facilitated by prior immersion of the embryo in $\sim$ 1 mL of 0.04% dispase in CMF for $\sim$ 1 min, after which the dispase was replaced with CMF.

Stage 11–14 donor embryos were pinned ventral side down on sylgard (Dow Corning, Belgium) in CMF. Lengths of donor neural tube, notochord and PSM were dissected, freed of adherent sclerotome cells using 0.04% dispase in CMF, and retained in sheep serum on ice before grafting into host embryos. The donor tissue was introduced into the host egg using a Gilson pipette and manoeuvred into position with the microscalpel. Donor PSM was taken from the right side of the donor embryo for grafting after anterior–posterior reversal into the left side of the host.

For grafts of somitocoele cells and epithelial somite cells, host embryos were prepared by making a cut between two adjacent epithelial somites (I–III) and between these somites and the neural tube/notochord. Donor embryos expressing cytoplasmic green fluorescent protein under the CAG promoter (CAG-GFP; McGrew et al. 2008; gift of Prof. Helen Sang) were pinned ventral side down in a sylgard dish. A cut was made between somites I and III and the notochord/neural tube in an anterior–posterior direction, removing the medial epithelial component of these somites. Somite strips were then pinned medial side up and a glass needle was used to remove the mesenchymal cells from the central somite cavity (somitocoele). These cells were

transferred into the prepared region of the host embryo using a Gilson pipette.

After grafting, 3–5 mL of albumen was removed from the host egg, which was then sealed using PVC tape. Embryos were incubated at 38 °C in a humidified incubator to the required post-operative stage, when they were pinned ventral side down on sylgard in Carnoy's solution (60% ethanol, 11.1% formaldehyde, 10% glacial acetic acid) overnight at 4 °C. For paraffin embedding, embryos were rinsed and dehydrated in 100% ethanol, immersed in Histosol (National Diagnostics, Atlanta, USA) until transparent (usually two 5-min washes), followed by immersion in three changes of paraffin wax (Fisher Scientific, Loughborough, UK) at 30-min intervals at 65 °C. Sections (10 μm) were cut using a rotary microtome (Microm), mounted in diethylpyrocarbonate (Sigma-Aldrich, Dorset, UK)-treated water on Superfrost Plus glass slides (VWR, East Grinstead, UK) and dried overnight.

RNA *in situ* hybridization

Full-length quail *Pax1* cDNA (gift of Dr Christine Ferguson; Ebensperger et al. 1995) was linearized with Xba1 and digoxigenin-labelled antisense RNA probes synthesized using the DIG RNA labelling kit with T3 RNA polymerase (Roche, Welwyn Garden City, UK), as described by the manufacturer. Purified labelled probes were reconstituted in hybridization buffer (50% formamide, 0.3 M NaCl, 10% dextran sulphate, 20 mM Tris pH 7.5, 5 mM EDTA, 10 mM sodium phosphate pH 8.0, 1 × Denhardt's, yeast tRNA 1 mg mL^{-1}) and used at a concentration of $\sim$ 1 ng mL^{-1}. Hybridization was carried out at 70 °C for $\sim$ 18 h using dewaxed 8-μm paraffin sections. After hybridization, slides were washed in 50% formamide, 1 × standard sodium citrate, 0.1% Tween-20 at 70 °C followed by four washes in MABT (0.1 M maleic acid, 150 mM NaCl, 0.1% Tween-20, pH 7.5). Slides were then blocked by incubation with MABT containing 20% sheep serum for 2 h before incubating with 1 : 2000 dilution of alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) in blocking buffer at room temperature for 18 h. After washing 5 × in MABT, slides were equilibrated with NTMT (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.1% Tween-20) before colour development using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt mix (Roche), according to the manufacturer.

Immunohistochemistry

After *in situ* hybridization, primary antibodies in PBS, 0.1% Triton X-100 (PBST), 10% sheep serum were applied and incubated at 4 °C overnight. After washing in PBST, slides were incubated with secondary antibodies for 3 h at room temperature, and then washed in PBST before mounting in Fluoromount-G. The following primary antibodies were used: GFP (rabbit; Invitrogen, Paisley, UK); TuJ1 (mouse IgG2a; Covance, Cambridge, UK); HNK1 (mouse IgM; gift of Dr Clare Baker); calretinin (rabbit; gift of Dr John Rogers); alexa 488- and alexa 594-coupled goat anti-mouse and goat anti-rabbit secondary antibodies (Invitrogen); and FITC-conjugated goat anti-GFP (Abcam, Cambridge, UK). All sections from each embryo were viewed using a Zeiss Axioskop microscope, and images taken using a Jenoptik C14 high-resolution colour camera with OPENLAB software (Improvision, Coventry, UK).

Results

Development of segmented regions of *Pax1* expression in the ventral sclerotome

The spatio-temporal pattern of *Pax1* expression during the early stages of somite development has been well documented in previous studies (see Discussion) and will not be described in detail here. Using *in situ* hybridization of embryo sections we confirmed that for maturing chick somites, at stages both preceding and during outgrowth of spinal axons, *Pax1* is strongly expressed throughout both anterior and posterior halves of the ventral sclerotome at the level of the notochord (Fig. 1A,B). With further maturation, expression becomes selectively more intense in the anterior half-sclerotome cells surrounding and medial to the spinal nerve components (Fig. 1C,D). By stage 29/30 the *Pax1*-expressing cells are localized into segmented groups or 'stripes' that extend medially from the posterior region of each segmented cluster of spinal nerve elements. Each stripe converges on the notochord, aligning with its counterpart on the opposite side and forming peri-notochordal rings in three dimensions (Fig. 1E, F). *Pax1* expression remains non-segmented and uniform in cells of the developing vertebral perichondrium ventral to the notochord, and by stage 36 expression in the ventral sclerotome of anterior segments has declined to undetectable levels (data not shown). No expression was detected in notochord cells at any of the stages examined.

In agreement with earlier studies, the anatomical positioning of the rings of *Pax1* expression indicates that they represent the developing annulus fibrosus, i.e. the component of the intervertebral disc that originates from sclerotome rather than notochord. Their lateral alignment with the developing spinal nerves, and their convergence on the notochord medially, raise the question whether their metameric repeat pattern originates from segmentally reiterated signalling from either or both of these structures, and this was investigated in subsequent experiments.

The notochord is dispensible for segmented *Pax1* expression in ventral sclerotome

We first tested whether the notochord is necessary for the development of segmented *Pax1* expression in the ventral sclerotome. As shown schematically in Fig. 2A, notochord excision was achieved by grafting a length of neural tube from a donor embryo (stage 11–14) into a host embryo of equivalent stage from which both notochord and neural tube had been excised at the same position as the donor. The operated region in the host embryo extended from the posterior PSM to the posterior (epithelial) somites, and was equivalent to 6–9 somites in length (Fig. 2A). Embryos were incubated to stage 29, after which *Pax1* expression was assessed by *in situ* hybridization of longitudinal sections.

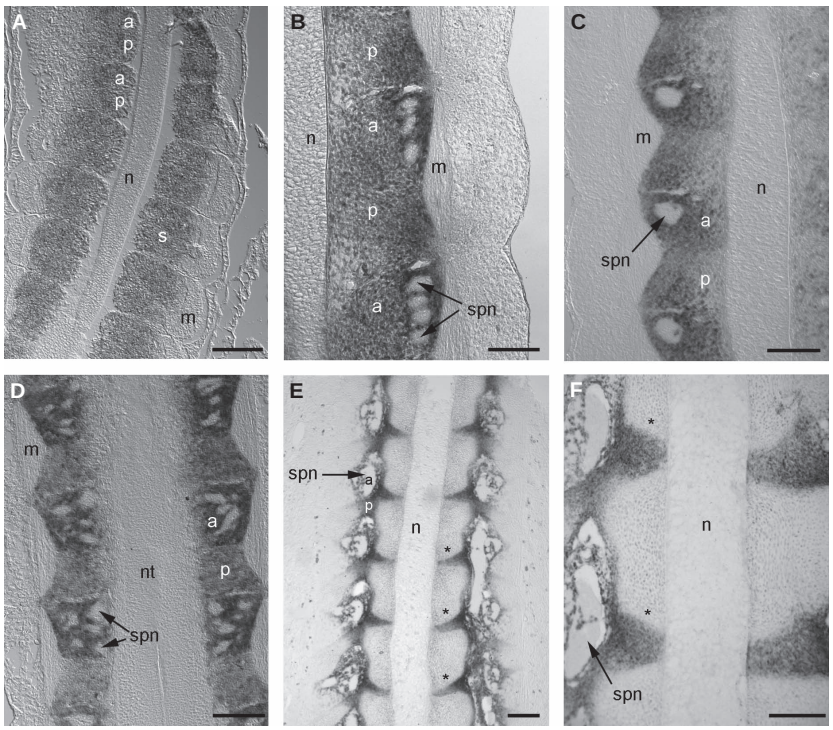

Fig. 1 *Pax1* expression in the developing chick sclerotome. Longitudinal sections, anterior upwards. (A) Stage 13 embryo at the level of the notochord, showing uniform *Pax1* expression in both anterior (a) and posterior (p) halves of each sclerotome. (B) Stage 24 embryo, thoracic somites; uniform *Pax1* expression persists in both sclerotome halves. (C, D) Stage 25 embryo, thoracic somites, at the level of the notochord (C) and neural tube (D), showing more intense *Pax1* expression in the anterior half-sclerotomes surrounding the spinal nerves. (E) Stage 29 embryo and (F) stage 30 embryo, showing resolution of *Pax1* expression into segmentally repeated stripes of cells (asterisks) extending medially from the spinal nerves and converging on the notochord. a, anterior half-sclerotome; m, myotome; n, notochord; nt, neural tube; p, posterior half-sclerotome; s, sclerotome; spn, spinal nerve. Scale bars: 100 μm.

Notochordectomized regions of sclerotome were readily identified in seven such embryos, and these regions showed segmented expression of *Pax1* laterally throughout their longitudinal extent, in association with the developing spinal nerves (Fig. 2B,C; Table 1). Levels of medial and midline *Pax1* expression in ventral sclerotome varied in these segments. In the absence of the notochord approximately a quarter (10/41 segments) showed only lateral *Pax1* expression (Fig. 2B), and half (23/41) showed interrupted stripes of *Pax1* expression that included cells at the midline (Fig. 2C). The remainder (8/41) showed segmented stripes of *Pax1* expression across the full width of the sclerotome (Fig. 2C), a pattern not seen in normal embryos. In cells of the most ventral sclerotome, *Pax1* expression was non-segmented and uniform, as in the ventral vertebral perichondrium of normal embryos (data not shown).

Further dorsal, at the level of the neural tube, all operated regions showed stripes of *Pax1* expression that were indistinguishable from those seen in normal embryos, again showing expression in association with the developing spinal nerves (data not shown). In summary, *Pax1* expression persists in a segmented pattern despite earlier removal of the notochord adjacent to the PSM, albeit at reduced levels in medial regions. While this argues against an absolute requirement for segmental notochord signalling in the generation of vertebral body/intervertebral disc segmentation, as noted above the result could also be explained if the remaining neural tube or spinal nerves impart segmental patterning information to the ventral vertebral column.

The neural tube is dispensible for segmented *Pax1* expression in ventral sclerotome

To test whether the neural tube and/or spinal nerves impart segmental signalling to the sclerotome, we assessed the consequences of neural tube excision in stage 11–14 embryos for segmented *Pax1* expression in the sclerotome. To ensure complete removal of the neural tube, including the floor plate, host embryos were prepared by excising a length of both neural tube and notochord, and replacing these with donor notochord from an embryo of the same developmental stage (Fig. 3A). The operated region was posterior to the second most recently-formed somite, and was equivalent to 6–12 somites in length, extending up to three-quarters of the length of the PSM. Embryos were incubated to stage 29, after which *in situ* hybridization for *Pax1* was performed on longitudinal sections.

Neuralectomized regions with intact grafted notochord were readily identified in 13 operated embryos, allowing assessment of a total of 80 sclerotomes. To exclude the possibility of neural crest migration into the operated region from either end of the remaining host neural tube, we also assessed the presence of spinal nerves in these segments using immunohistochemistry for TUJ1 and HNK1. TUJ1 labels class III beta tubulin (Caccamo et al. 1989), which is specific to neurons, while HNK1 labels a carbohydrate epitope expressed by migratory neural crest cells and by the crest-derived neuronal and glial lineages of the peripheral nervous system (Vincent & Thiery, 1984). Four embryos were additionally assessed for HNK1 staining 52 h post-operation,

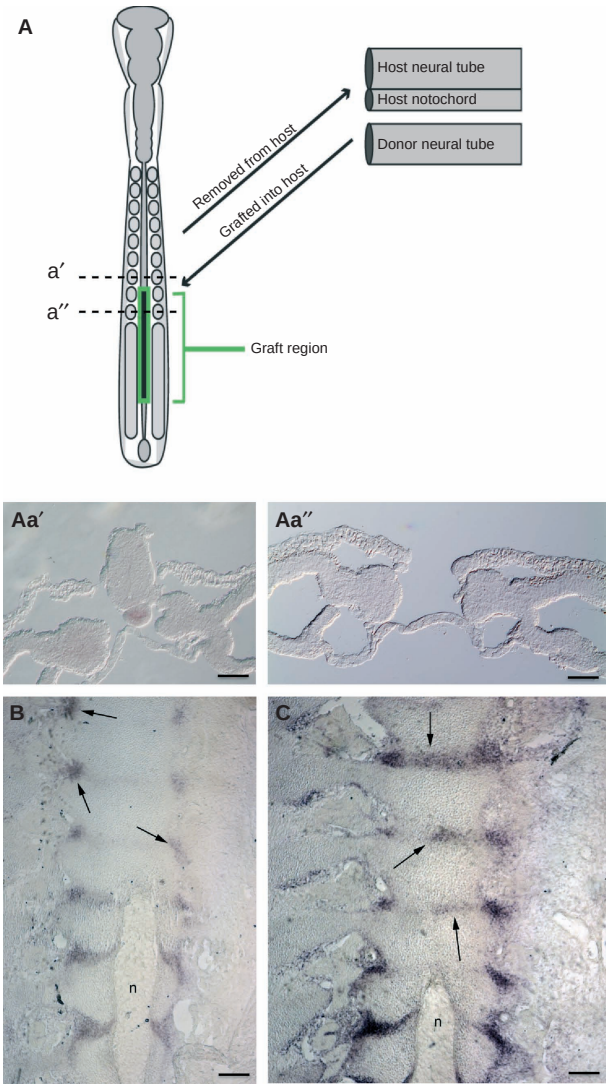

Fig. 2 Notochord excision. (A) Schematic diagram showing experimental strategy for notochordectomy. The dashed lines (a′ and a″) show the levels of the sections shown in (Aa′) and (Aa″). (Aa′) Transverse section through the unoperated region of an embryo fixed immediately after excision of the neural tube and notochord, and processed for *shh in situ* hybridization; *shh* expression (red) is visible in the notochord. (Aa″) Transverse section of the same embryo through the excised region and processed for *shh in situ* hybridization; removal of the notochord and its sheath is confirmed, and the midline endoderm remains intact. (B) Longitudinal section of a stage 29 host embryo showing the unoperated region with notochord (posterior, downwards) and the notochordectomized region (anterior, upwards). *Pax1* expression remains segmented in the grafted region anterior to the notochord (arrows), being conspicuous laterally but diminished medially in comparison with the stripes of expression seen alongside the notochord in the unoperated region. (C) Similar section to (B) in another notochordectomized embryo, showing persistent *Pax1* expression both laterally and medially (arrows) in the grafted region anterior to the notochord. n, notochord. Scale bars: 100 μm.

and all lacked HNK1 staining in the operated sclerotomes, except for those located at the operated/unoperated boundaries (data not shown).

Table 1 Notochord excision and *Pax1* expression in the sclerotome.

Pax1 expression: lateral only	*Pax1* expression: both lateral and medial but interrupted	*Pax1* expression: across sclerotome not interrupted
10/41 segments Seven embryos	23/41 segments Seven embryos	8/41 segments Seven embryos

Figure 3 shows TUJ1 and HNK1 staining in the unoperated (Fig. 3B–D) and operated (Fig. 3E–G) regions of the same embryo. TUJ1 and HNK1 staining was prominent in the unoperated regions containing the spinal nerves. Some notochord cells also stain for HNK1 at this stage of development, and were visible in both unoperated and operated regions of this embryo. However, no TUJ1- or HNK1-staining, or morphologically distinct spinal nerves were present in the operated region. TUJ1/HNK1-stained spinal nerves were detected in only 5 of the 80 segments identified as lacking neural tube. As anticipated they were exclusively located at the boundaries of the unoperated and operated regions, and these segments were excluded from further analysis.

Despite the absence of the neural tube and morphologically distinct spinal nerves, 95% (76/80) of sclerotomes in the experimental embryos showed typical segmented stripes of *Pax1* expression alongside the notochord when compared with unoperated sclerotomes (Fig. 3H). Abnormal segmentation of *Pax1* expression was occasionally seen around the end of the grafted notochord (4/80 segments, 2/13 embryos), where it was deformed post-operatively (data not shown).

Segmented expression of *Pax1* can be experimentally misaligned at the midline by perturbing left/right alignment of the somites

The results of notochordectomy and neuralectomy indicate that neither the notochord nor the neural tube and developing spinal nerves are likely to provide signals that regulate segmental patterning of *Pax1* expression in the ventral sclerotome. We therefore tested whether segmentation is entirely intrinsic to the sclerotome and not susceptible to correction by putative external signals. This was achieved by reversing the anterior part of the PSM along the anterior–posterior axis on one side of the embryo, so misaligning the sclerotomes on left and right sides of the embryo, and assessing whether this affects left/right alignment of segmented groups of sclerotome cells expressing *Pax1* adjacent to the notochord.

Figure 4A shows the resulting misalignment at the midline in the dorsal (neural arch) region of the sclerotome (stage 29), where the dorsal root ganglia and developing vertebral pedicles are out of register when comparing

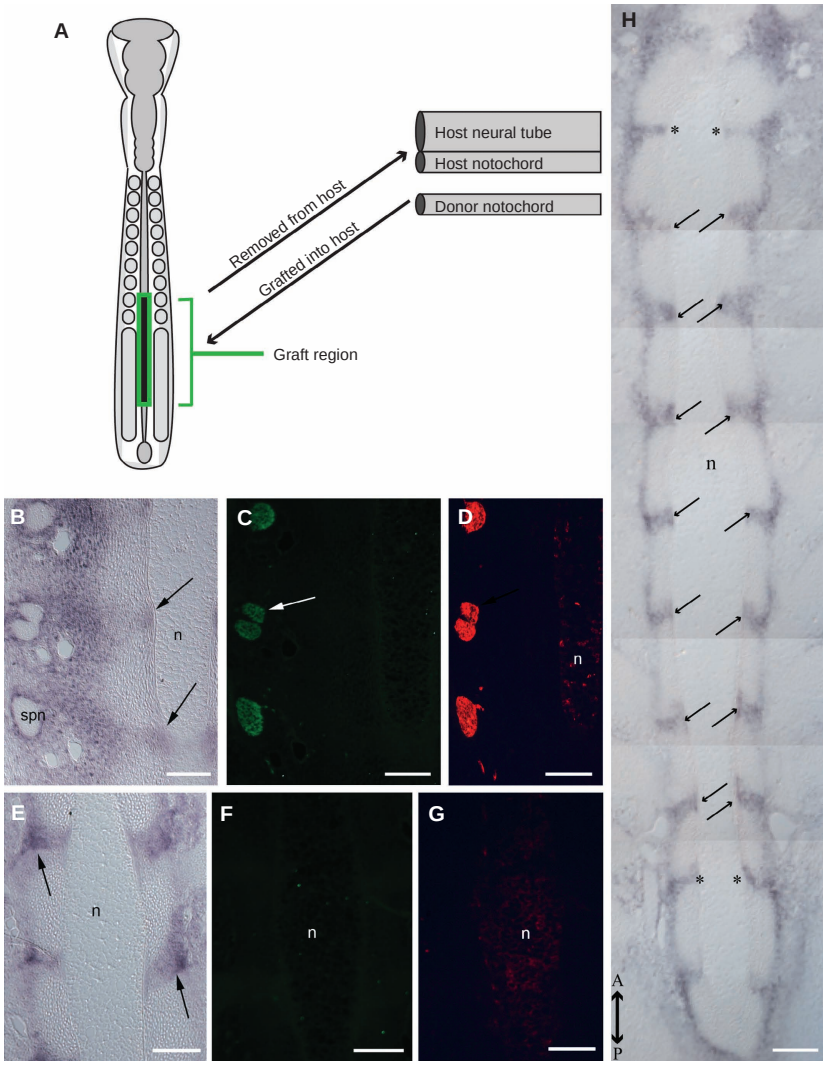

Fig. 3 Neural tube excision. (A) Schematic diagram showing experimental strategy for neuralectomy. (B–D) Longitudinal sections (anterior upwards) of the unoperated region of a stage 29 host embryo. *Pax1* expression is shown in a bright-field image (B), present in two horizontal stripes (arrows) adjacent to the notochord, and in the vicinity of the spinal nerves. Spinal nerves also stain for TUJ1 (C; arrow) and HNK1 (D) expression, and some notochord cells also stain weakly for HNK1. (E–G) Longitudinal sections (anterior upwards) of neuralectomized region at the level of the notochord, showing stripes of *Pax1* expression (arrows) adjacent to the notochord (E), in the absence of TUJ1 and HNK1 expression (F, G) confirming removal of spinal nerve components; weak HNK1 expression persists in notochord cells. (H) Composite longitudinal section at the level of the notochord along the entire length of the operated region of a neuralectomized embryo, anterior (A) and posterior (P) directions as indicated. *Pax1* expression (arrows) remains segmented at the level of the notochord. Stripes were occasionally somewhat thicker along the anterior–posterior axis than in normal embryos, perhaps indicating that the neural tube and/or spinal nerves influence overall growth of the annulus fibrosus. The segments marked with an asterisk lie at the boundaries of the operated and unoperated regions. n, notochord; spn, spinal nerve. Scale bars: 100 μm.

host and donor sides. Left/right misalignment of *Pax1* stripes was assessed in ventral sections at the level of the notochord, allowing for minor left/right midline misalignments present in normal embryos; misalignment was scored when there was no overlap between left and right *Pax1* stripes where they are adjacent to the notochord. The non-operated segments in eight experimental embryos showed only one *Pax1* stripe out of 50 assessed that misaligned at the midline, and none showed lateral misalignment. In contrast, misalignment was present in the reversed regions of all eight experimental embryos, and 80% (36/45) of reversed segments showed *Pax1* stripes that misaligned at both the midline and laterally (Fig. 4B). The characteristic left–right curvature of the *Pax1* stripes in grafted segments was also reversed (Fig. 4B). No stripe misalignment was detected in three sham-operated embryos in which the grafted PSM was first removed from the host and then replaced isotopically (data not shown).

Somitocoele cells acquire new fates when grafted into ectopic positions

The results above support the possibility that segmental patterning of vertebral bodies and intervertebral discs originates entirely in the somite mesoderm and is not influenced by external signals. The question then arises, at what stage during somite development is body/disc segmentation irreversibly determined? Quail/chick lineage analysis has shown that the sclerotome contribution to the intervertebral disc (future annulus fibrosus) is from somitocoele cells lying in the cavity of the newly-formed epithelial somite, raising the possibility that body/disc segmentation is determined as early as the initial stage of overt somite formation from the PSM (Huang et al. 1994, 1996; Mittapalli et al. 2005). We therefore assessed whether somitocoele cells acquire their fates irreversibly while resident in the epithelial somite. GFP-expressing donor somitocoele cells derived from GFP-transgenic embryos were grafted ectopically into wild-type

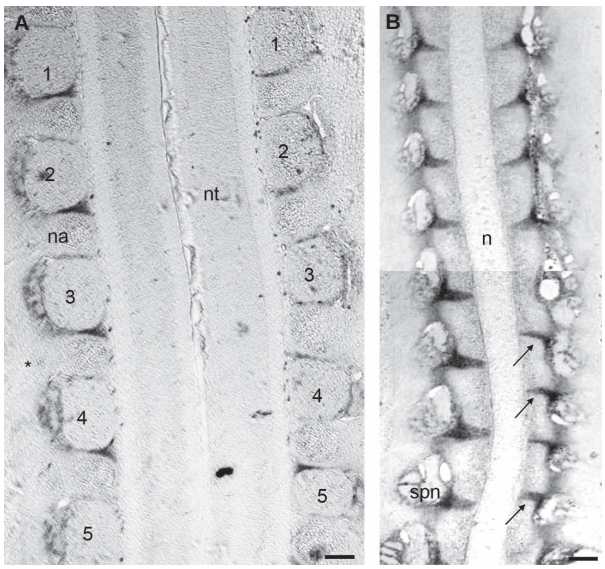

Fig. 4 Reversal of PSM unilaterally along the anterior–posterior axis. (A) Longitudinal section through stage 29 host embryo at the level of the neural arches and neural tube, anterior upwards. Numbers indicate individual dorsal root ganglia, and the intervening neural arches (pedicles) are placed between them. Ganglia 3, 4 and 5 show left/right misalignment resulting from three-segment PSM reversal on the right-hand side. (B) Longitudinal section through a stage 29 host embryo at the level of the notochord, showing left/right misalignment of the stripes of *Pax1* expression (arrows) in the region of PSM reversal. The characteristic curvature of the stripes is also inverted in the reversed region compared with the four normal segments placed more anteriorly on the right-hand side and with those on the left-hand side. Size differences between normal and reversed regions (e.g. for ganglia, neural arches and distances between adjacent *Pax1* stripes) may reflect overall growth differences between these regions in the graft vs. host. n, notochord; na, neural arch; nt, neural tube; spn, spinal nerve. Scale bars: 100 μm.

hosts, between two adjacent epithelial somites (I–III) and between these somites and the neural tube/notochord. Embryos were allowed to develop to HH stage 29–31, followed by section-*in situ* hybridization for *Pax1* and immunohistochemistry for GFP and calretinin, the latter as a marker for developing annulus fibrosus cells (Gangji et al. 1994). The results were compared with those seen when grafting epithelial somite cells into a similar ectopic location in wild-type hosts.

Five embryos survived unilateral ectopic grafting of GFP-expressing somitocoele cells, and three survived unilateral ectopic grafting of GFP-expressing epithelial somite cells. All eight embryos showed normal segmental expression of *Pax1*, and all of the five embryos that were successfully stained for calretinin expression (three somitocoele cell grafts and two epithelial cell grafts) also showed normal segmental expression of this marker. There was no ectopic expression of either *Pax1* or calretinin in the vertebral bodies of these embryos, and all *Pax1* stripes in GFP-positive regions aligned with control stripes on the unoperated side.

Figure 5 shows representative longitudinal sections from embryos with ectopic somitocoele or somite epithelial cell grafts. No ectopic *Pax1* or calretinin expression was visible in the vertebral bodies (Fig. 5A,B,E,F), nor were ectopic GFP-expressing somitocoele or epithelial cells restricted to either the intervertebral disc or the vertebral body (Fig. 5C,D,G,H); somitocoele and epithelial cells contributed to both vertebral body and intervertebral disc lineages. Moreover, in all embryos with somitocoele grafts some GFP-expressing cells migrated away from the ventral vertebral column to surround the developing spinal nerves (Fig. 5A,I,J), and contribute to the myotome (Fig. 5K,L) and neural arches (data not shown). These results indicate that the fates of somitocoele cells and epithelial somite cells are not irreversibly determined at the earliest stages of somite development, and can be modified following grafting to ectopic positions.

Discussion

The spatio-temporal changes in *Pax1* expression during vertebral development, from uniform expression in the peri-notochordal sclerotome to a later segmented pattern, were well documented in the first studies of *Pax1* expression in the mouse (Deutsch et al. 1988). These authors noted that as development proceeds, *Pax1* expression becomes localized to the sclerotome-derived component of the developing intervertebral disc, the future annulus fibrosus, being downregulated in the developing vertebral body/centrum, a pattern subsequently confirmed for *Pax1* protein (Wallin et al. 1994). *Pax1* expression is absent in notochord cells throughout development (Wallin et al. 1994).

Our study using section-*in situ* hybridization in the chick embryo shows a similar pattern of *Pax1* expression, as also described by Peters et al. (1995), and we find that expression becomes selectively intense in the anterior half-sclerotome cells surrounding the peripheral nerve elements, as noted for the mouse by Wallin et al. (1994) and the quail by Ebensperger et al. (1995). In contrast, some studies have detected stronger *Pax1* expression in the posterior compared with anterior half-sclerotome in both mouse and chick (Koseki et al. 1993; Barnes et al. 1996; Baffi et al. 2006), which may reflect the use in these studies of whole-mount *in situ* hybridization and the greater density of cells in posterior vs. anterior half-sclerotome. Taken together, there is a consensus that the persisting rings of peri-notochordal *Pax1* expression pre-figure the intervertebral discs, and are necessary for their development (Wallin et al. 1994; Dietrich & Gruss, 1995). *Pax1* expression has also been shown to persist throughout maturation of the rat annulus fibrosus (DiPaola et al. 2005). Accordingly we have used the *Pax1* expression pattern as a convenient assay for segmental patterning during the later stages of vertebral development.

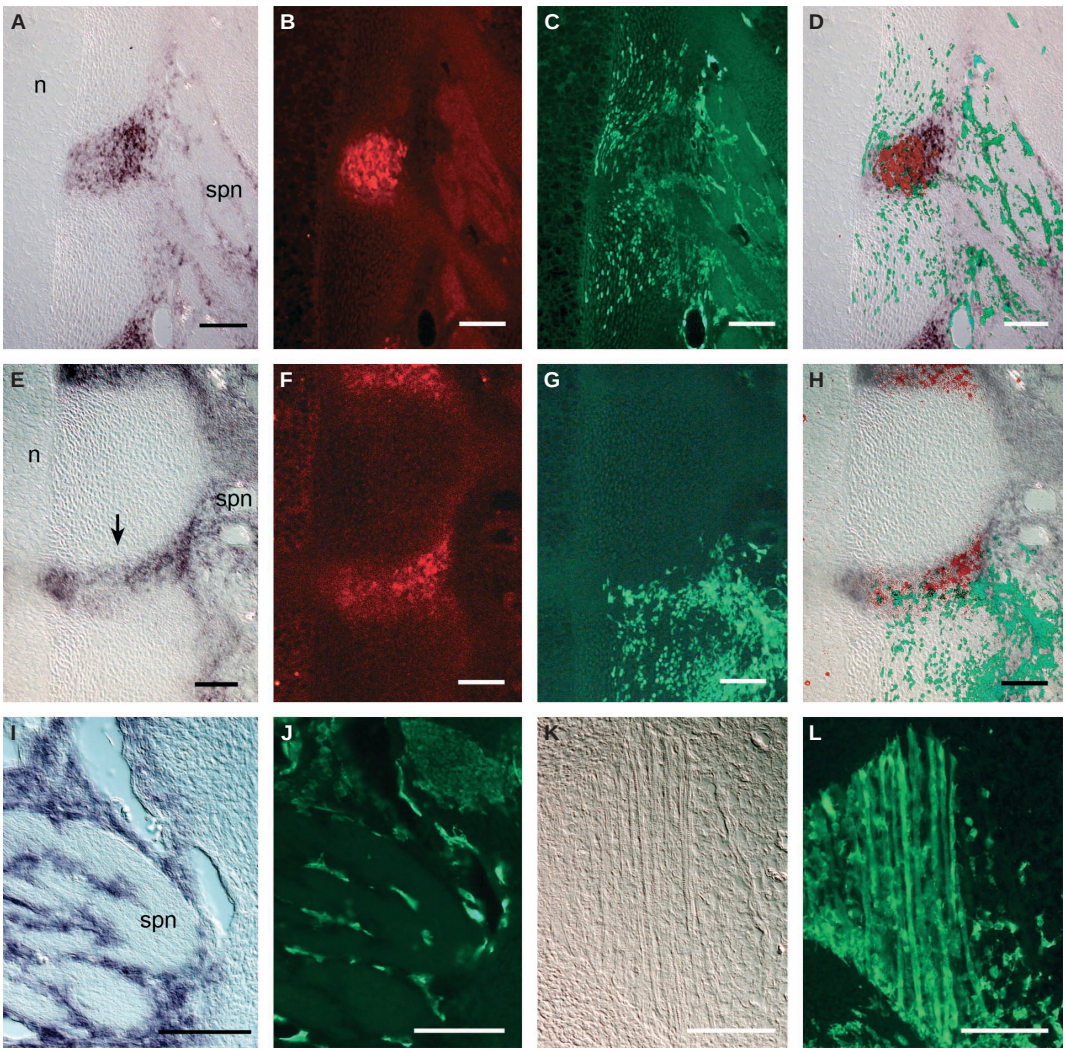

Fig. 5 Ectopic grafts of GFP-labelled somitocoele cells and epithelial somite cells. (A–D) Stage 31 embryo, longitudinal section after an ectopic graft of somitocoele cells between epithelial somite III and the neural tube/notochord. (A) Typical striped *Pax1* expression in the graft region between the notochord and spinal nerves, as in normal embryos; (B) expression of calretinin in the *Pax1*-expressing stripe in the same section. (C) The wide distribution of grafted GFP-labelled cells in this section; (D) an overlay of (A–C). Despite extensive dispersal of grafted somitocoele-derived cells, *Pax1* and calretinin expression show normal localization with no evidence of ectopic expression. (E, F) Stage 29 embryo, longitudinal section after an ectopic graft of somite epithelial cells between epithelial somite III and the neural tube/notochord. (E) Typical striped *Pax1* expression in the graft region between adjacent vertebral bodies; (F) calretinin expression in the same stripe of cells. (G) Extensive dispersal of grafted GFP-labelled cells in this section; (H) the overlay of (E–G). Grafted epithelial cells are dispersed widely in both vertebral and intervertebral positions. (I–L) Stage 31 embryo, longitudinal sections after an ectopic graft of somitocoele cells between epithelial somite III and the neural tube/notochord. *Pax1*-expressing cells (I) and GFP-labelled cells (J) occupy positions adjacent to spinal axons; GFP-labelled cells also contribute to the myotome (K, bright-field; L, GFP expression). spn, spinal nerve. Scale bars: 100 μm.

The classical experiments of Kitchin (1949) and Holtzer (1952b) in amphibian embryos, and Watterson et al. (1954) and Strudel (1955, 1967) in chick embryos, showed that early notochord excision results in a non-segmented column of cartilage extending ventral to the neural tube in the region of the notochordectomy, while dorsal vertebral (neural arch) segmentation persists. This could be taken as evidence that the notochord is required for primary segmental patterning of the ventral vertebral column. However, the notochord is well known to contribute the cells of the nucleus pulposus (Balfour, 1881; Peacock, 1951; Walmsley, 1953; Urban et al. 2000; Choi et al. 2008). It also provides a primary source of sonic hedgehog (Shh; Fan & Tessier-Lavigne, 1994; Johnson et al. 1994; Teillet et al. 1998) and the BMP antagonists Noggin (Hirsinger et al. 1997; Marcelle et al. 1997; McMahon et al. 1998) and gremlin1 (Stafford et al. 2011) that promote expression of *Pax1* and other sclerotome genes in the adjacent sclerotome (Christ et al. 2007; Chal & Pourquié, 2009). Its early removal will therefore preclude the appearance of the nucleus

pulposus, and the associated reduction in *Pax1* expression may be insufficient for normal development of the annulus fibrosus. The loss of macroscopic intervertebral segmentation seen in the classical studies can therefore be explained without the need to invoke a primary segmental patterning system derived from the notochord, being driven instead by segmentation in the somite mesoderm. In support of this interpretation, the present study shows that segmented *Pax1* expression ventral to the neural tube is still detectable in notochordectomized embryos. This is likely to result from the residual production of Shh and BMP antagonists by the floor plate, as is the persistence of *Pax1* expression in more dorsal sclerotome at the level of the neural tube.

Segmental *Pax1* expression also persists laterally in notochordectomized embryos in the vicinity of the segmented spinal nerves, even when medial expression is lost. This raises the possibility that signalling from the spinal nerve components (neural crest cells and axons) and/or neural tube may contribute to ventral vertebral segmental patterning, in addition to the well-known influence of the crest-derived dorsal root ganglia on dorsal vertebral (neural arch) segmentation (Holtzer, 1952a; Strudel, 1955, 1967; Hall, 1977; Schrägle et al. 2004; Colbjorn Larsen et al. 2006). However, Strudel (1955) noted that the peri-notochordal cartilage forming after neuralectomy in the chick embryo does show signs of macroscopic segmentation, and our findings are in agreement with this. Complete removal of the neural tube at a stage preceding earliest crest emigration (Rickmann et al. 1985), in the absence of signs of neural crest migration into the operated region from either end of the remaining host neural tube, does not prevent the appearance of segmental stripes of ventral, peri-notochordal *Pax1* expression. The neuralectomy extended to a position opposite the posterior PSM, suggesting that if the neural tube does nonetheless impart segmental patterning to the ventral sclerotome, it must arise very early in the formation of the somite mesoderm. It was also noted that segmented *Pax1* stripes in neuralectomized embryos were occasionally somewhat thicker along the a–p axis than in normal embryos, perhaps indicating that the neural tube and/or spinal nerves may influence the growth of the annulus fibrosus.

The further finding that left/right alignment of segmented peri-notochordal *Pax1* expression can be disrupted by unilateral PSM reversal also argues strongly against the possibility that vertebral segmental patterning originates in the notochord. At least up to stage 29 there is no indication that the notochord determines the reiterated positions of the adjacent *Pax1* stripes, which must instead result entirely from signals intrinsic to the somite mesoderm (see below). A similar lack of left/right registration of intervertebral discs has been noted by Goldstein & Kalcheim (1992) after unilateral grafts of multiple anterior half-somites from quail donors in chick embryo hosts. A plausible model is that segmentally positioned *Pax1*-expressing cells signal to adjacent notochord cells to position the nucleus pulposus, for example by promoting local notochord cell survival through TGF-β signalling (Baffi et al. 2006; Sohn et al. 2010). Rather less plausibly, nucleus pulposus segmentation could result from a late-expressed and autonomous property of the notochord whose period coincidentally matches that of the *Pax1* stripes, correlating with persistent *shh* expression seen in nucleus pulposus cells (DiPaola et al. 2005). It is notable that notochord *shh* expression is not segmented during the stages when *Pax1* expression becomes segmental, so (unless subject to periodic post-transcriptional processing) it is unlikely to provide the primary segmental pattern. On the other hand, Shh signalling has been shown to be essential for subsequent intervertebral disc formation, for example by maintaining the integrity of the notochordal sheath (Choi & Harfe, 2011) and promoting the movement of notochord cells into intervertebral positions (Aszodi et al. 1998). A similar argument against primacy in segmental patterning applies in the case of Wnt4b signalling, which is essential in generating medaka vertebral segmentation but is expressed non-segmentally in the floor plate (Inohaya et al. 2010).

Mittapalli et al. (2005) have proposed that the mesenchymal cells in the cavity of the avian epithelial somite (somitocoele) form a distinct joint-forming 'compartment' (arthrotome) that is fated to give rise to the intervertebral joints and discs, as well as to the ribs. Consistent with this, they have shown that experimental ablation of these cells prevents normal development of intervertebral joints and discs. In the present study we find that ectopically transplanted GFP-labelled somitocoele cells can contribute to other somite-derived structures, such as vertebral bodies and myotome, according to their new position, with no evidence of ectopic expression of markers of intervertebral disc fate (*Pax1*, calretinin). This suggests that if somitocoele cells normally generate intervertebral joint-related cells, this fate is not irreversibly determined at the somitocoele stage. Lineage tracing using quail–chick chimeras has shown that during anterior–posterior polarization of the sclerotome, the majority of somitocoele cells localize in the posterior (caudal) half of the sclerotome, adjacent to the intrasclerotomal anterior–posterior boundary (Huang et al. 1994, 1996; Christ et al. 2007). This finding is apparently at odds with the observation of Goldstein & Kalcheim (1992) that intervertebral disc fate correlates with anterior rather than posterior half-somites, unless their grafts of anterior halves, made at the epithelial stage of somite formation, more usually included the somitocoele cells. In either case, however, it is possible that local signalling interactions at the intra-segmental boundary between anterior and posterior somite halves, perhaps initiated even before overt somite formation, generate both the somitocoele cells and, ultimately, the segmented intervertebral joint components. The role of cell–cell interactions at segment and compartment boundaries in generating new signalling centres, and hence new cell fates, is well recognized in other regions of the

embryo, such as vertebrate hindbrain rhombomeres and *Drosophila* imaginal discs (Kiecker & Lumsden, 2005; Dahmann et al. 2011).

As already noted, studies in at least two teleost species, the zebrafish and Atlantic salmon, indicate that the chordacentra of the vertebral bodies are derived from segmental osteogenic activity in the notochord (Fleming et al. 2001, 2004; Grotmol et al. 2003, 2005). This implies that, alongside the somite mesoderm, the notochord is also intrinsically segmented in these species, although segmental patterns of gene expression in the notochord have yet to be described. Indeed, Stern (1990) has suggested that the notochord may be the archetypal segmented structure in the vertebrate trunk, perhaps including basal chordates and, if so, our results argue that this has been lost in the transition to reptiles, birds and mammals. Alternatively, if somite segmentation is archetypal, notochord segmentation may have been superimposed during teleost evolution as a derived character, perhaps to generate chordacentra whose segmental positions can vary with respect to the neural and haemal arches (Lauder, 1980). Accordingly, it will be interesting to investigate whether the notochord imparts centrum segmentation in more basal vertebrates such as the elasmobranchs.

At all events our study confirms in the chick the primacy of the somite mesoderm in generating vertebral segmentation, and we argue that the anterior–posterior polarity of the sclerotome is critical here. Consistent with this, a variety of mouse knockouts of genes involved in establishing and maintaining somite polarity show disrupted vertebral segmentation. These include the anterior determinant *Mesp2* (Saga et al. 1997), the posterior determinant *presenilin1* (Shen et al. 1997; Wong et al. 1997), and genes such as *paraxis* (Johnson et al. 2001), *Dll3* (Kusumi et al. 1998; Dunwoodie et al. 2002) and *Hes7* (Bessho et al. 2001) that also coordinate somite polarity. Alongside boundary-based signalling, we suggest that the subdivision of each sclerotome into non-miscible, and hence non-motile, anterior and posterior halves also serves an additional function. Vertebral development from migratory mesenchymal sclerotome cells can occupy several days in amniote embryos. During this process, therefore, the parcellation of the sclerotomes into non-miscible subunits provides a robust mechanism to preserve both the segmental pattern along the long axis and its left/right alignment at the axial midline.

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Author contributions

B.S.: design, data collection and analysis, critical review; C.S.: data collection; D.T.: concept and design, data analysis, critical review and approval of manuscript; R.K.: concept and design, data collection and analysis, draft and approval of manuscript.

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