A central role for the notochord in vertebral patterning

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Summary

The vertebrates are defined by their segmented vertebral column, and vertebral periodicity is thought to originate from embryonic segments, the somites. According to the widely accepted ‘resegmentation’ model, a single vertebra forms from the recombination of the anterior and posterior halves of two adjacent sclerotomes on both sides of the embryo. Although there is supporting evidence for this model in amniotes, it remains uncertain whether it applies to all vertebrates. To explore this, we have investigated vertebral patterning in the zebrafish. Surprisingly, we find that vertebral bodies (centra) arise by secretion of bone matrix from the notochord rather than somites; centra do not form via a cartilage intermediate stage, nor do they contain osteoblasts. Moreover, isolated, cultured notochords secrete bone matrix in vitro, and ablation of notochord cells at segmentally reiterated positions in vivo prevents the formation of centra. Analysis of fss mutant embryos, in which sclerotome segmentation is disrupted, shows that whereas neural arch segmentation is also disrupted, centrum development proceeds normally. These findings suggest that the notochord plays a key, perhaps ancient, role in the segmental patterning of vertebrae.

Key words: Segmentation, Somites, Notochord, Vertebræ, Bone, Zebrafish

Introduction

The segmented vertebral column is the defining feature of the vertebrates, and is composed of an alternating pattern of bony vertebral bodies (centra) and intervertebral discs. In birds and mammals, it is well known that the centra originate from the somite-derived sclerotomes, and the molecular basis for sclerotome differentiation is beginning to be elucidated. For example, sonic hedgehog (Shh) signalling (Fan et al., 1995; Fan and Tessier-Lavigne, 1994) and several transcription factors, including members of the Pax and Sox families (Akiyama et al., 2002; Balling et al., 1996; Smits and Lefebvre, 2003) and Bapx (Lettice et al., 1999; Triebold and Lufkin, 1999), have been found to be required for sclerotome differentiation and proliferation (for a review, see Christ et al., 2000).

Despite these insights at the molecular level, the relationship between somite patterning and vertebral segmentation remains to be firmly established. Somites give rise to both axial muscles (from the myotome) and vertebrae (from the sclerotome) but, as Remak first observed, vertebrae are displaced by half a segment relative to muscle segments (Remak, 1850). To account for this frameshift, he proposed that a single vertebra is formed from a combination of the anterior (cranial) half of one sclerotome with the posterior (caudal) half of the next-anterior sclerotome (Remak, 1850; Verbout, 1976). Thus a single somite gives rise to a single muscle element yet contributes to two adjacent vertebrae. This ‘resegmentation’ model has been widely accepted for amniotes, both from early descriptive studies (e.g. Goodrich, 1930; Gadow, 1933) and more recent cell lineage studies (e.g. Bagnall et al., 1988; Goldstein and Kalcheim, 1992; Huang et al., 1996; Aoyama and Asamoto, 2000; Huang et al., 2000). Resegmentation has remained somewhat controversial nonetheless (Verbout, 1976; Keynes and Stern, 1988), and it has also been unclear whether the model is applicable to all vertebrates. In zebrafish, sclerotome cells constitute only a small proportion of the somite (Stickney et al., 2000) and can give rise to muscle (Morin-Kensicki and Eisen, 1997). Moreover, a recent lineage analysis in the fish has shown that cells from one half-sclerotome can contribute to two consecutive vertebrae, rather than only to one vertebra as predicted by resegmentation (Morin-Kensicki et al., 2002). This has been termed ‘leaky’ resegmentation, and is consistent with a similar finding made in a previous study using the chick embryo (Stern and Keynes, 1987).

If the classical resegmentation model does not apply in zebrafish, the question remains as to the source of segmental patterning of centra in this species. In particular, although the notochord is known to be critical for sclerotome development, its possible participation in the segmental patterning of vertebrae has been neglected, and it has generally been regarded as a passive player in this process. With this in mind, we have therefore undertaken a study of zebrafish vertebral patterning, and our results suggest that the notochord plays a central role in segmental patterning.

Materials and methods

Maintenance of stocks and collection of embryos

Fish were reared under standard conditions (Westerfield, 1995). Embryos were collected from natural spawnings and staged according to established criteria (Kimmel et al., 1995). F_{S_4}^{ts14s}
mutant adults (van Eeden et al., 1996) were maintained as homozygous stocks.

**Skeletal staining**
Embryos and larvae were fixed in 4% paraformaldehyde (PFA), then labelled with alcian green and alizarin red as described (Kelly and Bryden, 1983). In vivo labelling was achieved by rearing fish in either 0.05% quercetin or alizarin red in embryo medium. Both quercetin and alizarin red were seen to label bone matrix; quercetin was found to be most suitable because of its low toxicity, utility at physiological pH and narrow fluorescence spectrum (Simmons and Kunin, 1979).

**Antibody and alkaline phosphatase labelling**
Larvae at 20 days post-fertilization (dpf), previously reared in alizarin red, were fixed in 4% PFA and stained in wholemount as described (Westerfield, 1995) with minor modifications. The zns5 monoclonal antibody (gift of Chuck Kimmel, University of Oregon, OR, USA) was used at 1:200 dilution; Alexafluor 488 (Molecular Probes, Eugene, OR) was used as a secondary antibody. Larvae were embedded in Sakura Tissue-Tek OCT Compound (Bayer, Newbury, UK) and frozen sections were cut at 10 μm thickness and counterstained using Vectashield containing DAPI (Vector Laboratories, Peterborough, UK). Sections were viewed using a Leica TCS-NT confocal microscope. Alkaline phosphatase staining was performed using an ELP97 cytolabelling kit (E6602) as described by the manufacturer (Molecular Probes) and visualised by fluorescence microscopy on a Zeiss Axioplan 2 microscope. With a DAPI filter set, the positive signal appears green. Centra at all axial levels were examined, and representative images were taken.

**Transmission electron microscopy**
Larvae at 20 dpf were fixed in 4% glutaraldehyde in cacodylate buffer containing 0.006% hydrogen peroxide for 3 hours, then washed in cacodylate buffer before post-fixing in osmium tetroxide. Samples were bulk stained with uranyl acetate, dehydrated in ethanol and embedded in Spurr’s resin. Thin sections (5 nm) were prepared with a Leica Ultracut UCT, stained with uranyl acetate and lead citrate and viewed in a Philips CM100 electron microscope at 80 KV.

**Notochord dissection**
Embryos at 2-6 dpf were anaesthetised in 0.2 mg/ml 3-amino benzoic acid ethylester (MS222) and then decapitated. Notochords were dissected using tungsten needles. Some were fixed immediately after dissection in 4% PFA and labelled with DAPI and/or 0.05% quercetin. Others were cultured in L-15 containing 10% FCS, 0.1% gentamycin at 32°C for 10-20 days before being labelled with quercetin and viewed using a Zeiss Axioplan 2 microscope.

**Notochord cell ablation**
Embryos and larvae were anaesthetised in 0.2 mg/ml MS222, mounted on a depression slide and viewed on a Zeiss Axioplan 2 microscope with Micropoint laser system (Photonic Instruments, St. Charles, IL, USA). Individual cells were ablated with pulses of 440 nm laser light. Targeted notochord cells were located either at the myoseptal border, or at the centre of the prospective centra; individual notochord cells are large and vacuolated, with a clear boundary, and are readily distinguished. Single cells were targeted with sub-micron resolution using the cross-hairs of the eyepiece (see Fig. 4C,D); targeted cells could be seen to burst, and surrounding undamaged notochord cells expanded to fill the available space. In most cases only one cell was ablated; cells outside the focal plane of the beam were not damaged because the beam energy was only sufficient to damage cells lying in the focal plane itself. Sometimes a second cell partially overlapped the targeted cell, and this was also ablated by a second laser pulse refocused on this second cell. Fish were allowed to recover and later labelled with 0.05% quercetin as described above.

**In situ hybridisation**
The Pax9 probe was a gift from E. Melancon and J. Eisen. The Smad1 probe was a gift from A. Dick and M. Hammerschmidt. In situ hybridisation was performed as described (Westerfield, 1995) with minor modifications.

**Results**
Zebrasfish bony centra do not pass through a cartilage intermediate stage
To establish the time-course of vertebral development, we employed alcian green and alizarin red to label cartilage and bone, respectively. Cartilage can first be detected in the craniofacial skeleton at ~2 dpf (data not shown), whereas the first cartilaginous elements associated with the vertebral column appear at ~7 dpf, being readily apparent at ~9 dpf (Fig.
This cartilage is only seen in the first four vertebrae, and forms the Weberian apparatus, a specialized structure that connects the swim bladder to the inner ear (Coburn and Futey, 1996; Morin-Kensicki et al., 2002). By 17 dpf, additional cartilaginous elements can be seen in the thoracic region, in the costal rib heads that articulate with the vertebral bodies (centra).

Strikingly, we find that centra remain devoid of cartilage in their transition to bone, with no intense staining comparable to that seen in the Weberian cartilage or the costal rib heads (Fig. 1C). Consistent with this, *coll2a1*-expressing chondrocytes are also absent from developing centra (data not shown). Ossification begins at ~7 dpf, and two days later the most anterior centra are readily distinguishable as bony cylinders surrounding the notochord (Fig. 1B). Centra are laid down in an anterior to posterior sequence (Fleming et al., 2001; Morin-Kensicki et al., 2002) and, by ~17 dpf, thoracic centra have ossified without passing through a cartilage stage (Fig. 1D). Ossified neural and haemal arches appear from ~17 dpf (Fig. 1D), and an essentially complete column is formed by 23 dpf (Fig. 1E,F). Because a cartilage intermediate is not observed at any stage, bony centra in zebrafish do not develop by endochondral ossification, which is in marked contrast to amniotes (Dockter, 2000).

**Osteoblasts are not present in centrum bone matrix**

A second mechanism of bone formation, intramembranous ossification, is recognized, where bone forms directly from mesenchymal cells that differentiate into osteoblasts, with no cartilage intermediate (Gilbert, 2000). We therefore looked for the presence of osteoblasts within the developing zebrafish centra. As a control we confirmed that zns5 (Johnson and Weston, 1995) and alkaline phosphatase (Simmons and Kunin, 1979) label osteoblasts in developing skull bones, where intramembranous ossification takes place (Fig. 2A,C). However, no osteoblasts were seen in the developing centra using these markers (Fig. 2B,D), suggesting that bone formation in developing centra is atypical. The thick section in Fig. 2D shows several nuclei in the notochord vicinity, and these probably arise from paraxial mesoderm and/or neural tube.

In some teleosts another, unusual mechanism of ossification has been noted, where bone matrix-secreting cells remain
external to the bone so that the latter is devoid of osteocytes (Ekanayake and Hall, 1987). We therefore examined centrum ossification by electron microscopy to determine whether a similar mechanism applies to zebrafish centra. Whereas the paraspinal bone displayed an appearance typical of intramembranous bone (Fig. 2E,G), the centrum comprised a thickened laminated matrix devoid of osteoblasts (Fig. 2F,H,I). It has been suggested that vertebral bone in another teleost, medaka, is deposited by osteoblasts lying outside the centra (Ekanayake and Hall, 1988). However, we find only muscle cells at this location in the zebrafish, implying that centrum matrix is secreted by the notochord in this species.

The notochord is a source of bone matrix in vitro

To investigate whether the notochord is a source of bone matrix, we dissected notochords free of surrounding tissues prior to centrum formation, and cultured them in vitro for 10-20 days, by which time centrum bone would have formed in vivo. Matrix deposition was assessed by labelling with quercetin (Simmons and Kunin, 1979). In cultured notochords strong labelling for bone matrix was seen between individual cells (Fig. 3A,B). Fully developed centra were not seen, presumably reflecting diffusion of matrix in vitro when the notochord is no longer confined by the presence of surrounding tissues. To exclude the presence of residual adherent osteoblasts on the outside surface of the notochord sheath, a nuclear label was also used, and no nuclei were seen external to the notochord (Fig. 3C-E).

The notochord is a source of bone matrix in vivo

If the notochord directs bone synthesis in vivo, laser ablation of notochord cells at defined locations might be expected to prevent centrum development. Because centrum bone deposition is initiated adjacent to the myoseptal border (Fig. 4A,B), we targeted single (or occasionally two – see Materials and methods) notochord cells at this location in the zebrafish, implying that centrum bone formation (4 dpf) cultured, and then labelled with quercetin for bone matrix (n=10). (A) After 12 days' culture, matrix is seen as bright transverse bands. (B) Bright field image of A using DIC optics; bone labelling is seen between cell boundaries. (C) Phase-contrast image of a notochord dissected and fixed at 4 dpf. (D) Composite phase-contrast and DAPI image of C showing nuclei (blue) only within the boundaries of the notochord sheath (adjacent to phase-bright lines); no cells are seen outside the sheath (n=8). (E) Notochord in C labelled with quercetin, showing no positive staining for bone matrix; the notochord is delineated by background fluorescence, and faint nuclear (DAPI) fluorescence is observed in the FITC channel. Scale bars: A,B, ~100 μm; C-E, ~80 μm.

An instructive role for the notochord in segmental patterning

According to the resegmentation hypothesis, disrupting somite segmentation should perturb vertebral segmentation, because the reorganization of half-sclerotomes would be abnormal. However, if the notochord also plays an instructive role in segmental patterning, its presence may be sufficient to maintain segmentation following somite disruption. In fused somites (fss) embryos, somites lose their anterior-posterior polarity and their overt boundaries are irregular (van Eeden et al., 1996). In these embryos, the neural and haemal arches are highly disorganized, but centra develop nonetheless with normal shape, size and periodicity (Fig. 5A-D) (see also van Eeden et al., 1996). This observation is consistent with a primary role for the notochord in centrum segmentation.

Some features of residual segmentation are known to be retained in fss mutants (van Eeden et al., 1996; van Eeden et al., 1998; Durbin et al., 2000; Nikaido et al., 2002), and it was therefore important to establish whether this is the case for sclerotome segmentation. We employed in situ hybridization with the sclerotome-specific markers, Pax9 (Nornes et al., 1996) and Smad1 (Dick et al., 1999), to assess sclerotome patterning in these mutants, and found that the segmental organization of the sclerotome is disrupted (Fig. 5E-H). Finally, we also confirmed the requirement for the notochord in centrum formation in fss embryos by laser ablation of notochord cells (Fig. 5I-J). Taken together, these observations...
suggest that, alongside the somites, the notochord also has a key role in imparting segmental information to the vertebral column.

**Discussion**

Our studies on the development of the zebrafish vertebral column have highlighted several unexpected features. First, although some sclerotome cells are known to contribute to vertebrae (Morin-Kensicki et al., 2002) (see below), we have found that the notochord secretes centrum bone matrix. Whether this is a more general mechanism for centrum formation in all vertebrates remains to be determined.

A previous morphological investigation of vertebral development in another teleost, medaka, has shown that bone matrix in this species is secreted by a layer of osteocytes that lie over the outer surface of the centrum, with no deposition on the inner surface (Ekanayake and Hall, 1987; Ekanayake and Hall, 1988). Matrix-producing cells avoid becoming trapped in their own secretion because of its polarized nature, where cells only generate matrix in the direction of previously deposited bone. We have found that zebrafish centra also lack osteoblasts embedded in the bone matrix, but the secretion process resembles an inverted version of that in medaka; matrix is deposited from inside the bony ring, by notochord cells, rather than from outside.

Such a mechanism raises the intriguing problem of how zebrafish centra increase in size during later development. One possibility is that the sclerotome-derived osteoblasts invade the centra at a later developmental stage. In keeping with this, the...
presence of some sclerotome cells has recently been noted in the developing zebrafish vertebra, particularly in the neural arches, showing that sclerotome cells can contribute vertebral bone (Morin-Kensicki et al., 2002). The relative contribution of sclerotome cells to centra is less clear, however, and requires further investigation.

**A duality in vertebral patterning**

Our results also suggest that the notochord may make an important contribution to the segmental patterning of the vertebral column. In particular, we find that segmentation is lost after ablation in vivo of notochord cells lying at precise, segmentally reiterated positions along the developing trunk, adjacent to the somite boundaries. This suggests that notochord cells adjacent to boundaries initiate centrum formation; subsequent expansion of the centrum may result from diffusion of this matrix along the long axis, and/or an additional contribution of matrix from neighbouring notochord cells at later stages.

In *fss* mutant embryos, where sclerotome patterning is abnormal, the subsequent patterning of the neural and haemal arches is also disorganized, yet the centra develop normally. In combination with the ablation studies, these findings suggest the existence of a duality in vertebral patterning, in which the notochord and somites impart complementary segmental information to the vertebral column. The notochord is necessary to generate the metameric arrangement of centra, whereas the somites direct patterning of the arch elements attached to them. It is interesting to note here that, like the centra, the arches also develop in the absence of a cartilage intermediate, implying that sclerotome-derived chondrocytes are also absent in this vertebral region. Although it is possible that residual manifestations of trunk segmentation may remain in *fss* mutant embryos (van Eeden et al., 1998), our use of these embryos to test the resegmentation hypothesis is appropriate. The key feature of this model is the...
subdivision of the sclerotome into anterior and posterior halves, with a subsequent recombination of adjacent halves; the finding that the segmental organization of the sclerotome is destroyed in fss mutants, yet centra are normal, presents a major difficulty for any simple version of this model.

A further difficulty in applying classical resegmentation in the zebrafish comes from a recent lineage study testing the hypothesis directly. Clonal analysis of the zebrafish sclerotome has shown that cells from one half-sclerotome can contribute to two adjacent vertebrae. There is, therefore, no tight correlation between sclerotome fate and vertebral origins in the zebrafish (Morin-Kensicki et al., 2002), and a similar finding has been reported previously using quail-to-chick half-somite grafts (Stern and Keynes, 1987). Morin-Kensicki et al. have proposed a ‘leaky’ resegmentation model, based on a continuing primacy for the sclerotome in vertebral patterning. This modified model does predict the extensive vertebral arch defects seen in fss mutants, but has greater difficulty in explaining the normal appearance of centra in these mutants. An alternative view, supported by the findings of this study, is that the notochord plays a central and instructive role in centrum segmentation.

Notochord segmentation

Our results suggest that the notochord is functionally segmented, raising the question of whether it is also segmented at the molecular level. To date, no periodic patterns of gene expression have been described in the early notochord, but these may exist nonetheless. Regionally restricted patterns of Hox gene expression have been described in the zebrafish notochord (Prince et al., 1998), suggesting that this morphologically uniform structure is at least subdivided regionally along the anterior-posterior axis. Further evidence for regional subdivision is provided by the observation that the anterior notochord in Xenopus is a more powerful inducer of En-2 in neural ectoderm than posterior notochord (Hemmati-Brivanlou et al., 1990); the regional inducing capacity of the early chondamesoderm is also well known (Spemann, 1938).

If the notochord has an instructive role in segmental patterning, the question arises as to how this is acquired. Segmentation of both notochord and somite mesoderm may originate simultaneously during gastrulation. Alternatively, notochord segmentation may be secondary to somite segmentation, being transmitted by unknown mechanisms between these adjacent tissues.

Overall, our results support the view that the notochord is an archetypal segmental structure in the vertebrate trunk (Stern, 1990; Fleming et al., 2001). Consistent with this view, extirpation of the notochord in amphibian and avian embryos results in the formation of a rod of vertebral cartilage lacking overt segmental features (Kitchin, 1949; Strudel, 1955). This raises the intriguing possibility that the notochord has a conserved role in the instruction of vertebral patterning in all vertebrate classes.

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