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Metameric pattern of intervertebral disc/vertebral body is generated independently of Mesp2/Ripply-mediated rostro-caudal patterning of somites in the mouse embryo

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ABSTRACT

The vertebrae are derived from the sclerotome of somites. Formation of the vertebral body involves a process called resegmentation, by which the caudal half of a sclerotome is combined with the rostral half of the next sclerotome. To elucidate the relationship between resegmentation and rostro-caudal patterning of somite, we used the *Uncx4.1-LacZ* transgene to characterize the resegmentation process. Our observations suggested that in the thoracic and lumbar vertebrae, the Uncx4.1-expressing caudal sclerotome gave rise to the intervertebral disc (IVD) and rostral portion of the vertebral body (VB). In the cervical vertebrae, the Uncx4.1-expressing caudal sclerotome appeared to contribute to the IVD and both caudal and rostral ends of the VB. This finding suggests that the rostro-caudal gene expression boundary does not necessarily coincide with the resegmentation boundary. This conclusion was supported by analyses of *Mesp2* KO and *Ripply1/2* double KO embryos lacking rostral and caudal properties, respectively. Resegmentation was not observed in *Mesp2* KO embryos, but both the IVD and whole VB were formed from the caudalized sclerotome. Expression analysis of IVD marker genes including *Pax1* in the wild-type, *Mesp2* KO, and *Ripply1/2* DKO embryos also supported the idea that a metameric pattern of IVD/VB is generated independently of Mesp2/Ripply-mediated rostro-caudal patterning of somite. However, in the lumbar region, IVD differentiation appeared to be stimulated by the caudal property and suppressed by the rostral property. Therefore, we propose that rostro-caudal patterning of somites is not a prerequisite for metameric patterning of the IVD and VB, but instead required to stimulate IVD differentiation in the caudal half of the sclerotome.

Introduction

The vertebrate axial skeleton is derived from the sclerotome of somites. Somites are paired metameric structures of paraxial mesoderm along both sides of the neural tube formed by segmentation of the presomitic mesoderm in regular temporal and spatial intervals. Newly formed somites begin to differentiate under the influence of surrounding tissues into the ventromedial sclerotome, which gives rise to the vertebrae and ribs, and the dorsolateral dermomyotome, which produces the skeletal muscles and dermis. A great deal of study has so far revealed the molecular mechanisms underlying various aspects of somitogenesis, including periodicity and regularity via a molecular clock, synchronization among cells, formation of the segment boundary, rostro-caudal patterning within each somite, and the mesenchymal-epithelial transition (Aulehla and Pourquie, 2008; Dequeant and Pourquie, 2008; Pourquie, 2007; Wahl et al., 2007). A series of studies on the bHLH transcription factor Mesp2 (Morimoto et al., 2006; Morimoto et al., 2005; Nomura-Kitabayashi et al., 2002; Oginuma et al., 2008; Saga et al., 1997; Sasaki et al., 2011; Takahashi et al., 2005; Takahashi et al., 2003; Takahashi et al., 2000; Takahashi et al., 2007b; Yasuhiko et al., 2006) and its downstream co-repressors Ripply1 and 2 have shown that the Mesp2/Ripply system plays critical roles in establishing the rostro-caudal patterning of each somite, as well as somite boundary formation (Hitachi et al., 2009; Kawamura et al., 2008; Moreno et al., 2008; Morimoto et al., 2007; Takahashi et al., 2010). Just prior to somite formation, one presumptive somite is subdivided into rostral and caudal halves, which differ in gene

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expression profile and subsequent cell differentiation. Mesp2 finally localizes to the rostral half of the somite and induces expression of rostral genes, such as *EphA4* and *Tbx18*, and suppresses Notch activity and the expression of caudal genes *Dll1* and *Uncx4.1*. Mesp2 also induces the expression of Ripply 1 and 2, which in turn play roles in restricting the expression domain of Mesp2 by suppressing the activity of Tbx6. In *Mesp2*-knockout (KO) embryos, the rostral half property is lost and instead the caudal half property is expanded throughout the somitic mesoderm (caudalization). In contrast, in *Ripply 1/2* double-knockout (*Ripply1/2* DKO) embryos, the caudal half property is completely lost and the rostral property is expanded (rostralization).

Though many studies have focused on the mechanism of somitogenesis and differentiation of the sclerotome, myotome, and dermatome, the mechanisms underlying subsequent vertebral morphogenesis are poorly understood (Christ et al., 2007). Previous studies have suggested that Pax1 and Pax9, which are part of the Pax family of transcription factors, are essential for formation of both the vertebral body (VB) and intervertebral disc (IVD) (Deutsch et al., 1988; Neubuser et al., 1995; Peters et al., 1999; Wilm et al., 1998). Specification of the pedicles of the neural arches, transverse processes, and proximal ribs requires the paired-domain transcription factor Uncx4.1 (we use herein *Uncx4.1*; the official gene symbol is now *Uncx*) (Leitges et al., 2000; Mansouri et al., 2000; Mansouri et al., 1997; Saito et al., 1996). At the dorsal-most vertebra, BMP and Msx are involved in the formation of spinous process (Monsoro-Burq et al., 1994; Monsoro-Burq et al., 1996).

In amniotes, a process called resegmentation occurs when the VB is formed from the sclerotome (Remak, 1855; von Ebner, 1889). That is, the sclerotome is subdivided into rostral and caudal halves at the sclerotomal fissure, or von Ebner's fissure, and the caudal half of a sclerotome is combined with the rostral half of the next sclerotome to form a VB. This scheme, which is illustrated in most developmental biology textbooks, naturally leads to the concept that a metameric pattern of somites is the basis for a metameric pattern of vertebrae, and the rostro-caudal patterning of each somite is essential for VB formation (Senthinathan et al., 2012). However, our previous observations suggested that segmentation of the VB does not necessarily reflect the status of somite segmentation. In *Mesp2* KO embryos, the somite boundary does not form and rostro-caudal patterning is lost, i.e. the caudal property expands throughout the somitic mesoderm. However, the VBs appear segmented, though irregular, whereas the pedicles of the neural arches and proximal ribs are almost completely fused (Saga et al., 1997; Takahashi et al., 2007a). In *Ripply 1/2* DKO embryos, the vertebral bodies appear segmented, and the pedicles of the neural arches and proximal ribs are completely lost (Takahashi et al., 2010). Thus, an obvious difference in severity exists between the medial and lateral elements of the vertebral column.

Although resegmentation has been experimentally studied in the chick embryo (Aoyama and Asamoto, 2000; Bagnall et al., 1988; Ewan and Everett, 1992; Huang et al., 2000; Huang et al., 1996), it has only been classically described in the mammalian embryo (Verbout, 1985). To elucidate the mode of resegmentation in mouse vertebrae, we analyzed the localization of caudal sclerotome-derived cells using *Uncx4.1-LacZ* transgenic mice. *Uncx4.1* is a reliable marker of the caudal half of somites. Expression of *Uncx4.1* initially localizes in the caudal half of each epithelial somite, then the caudal half of the sclerotome, and finally in the caudal lateral sclerotome giving rise to the pedicle of the neural arch (Mansouri et al., 1997). Therefore, *Uncx4.1* is transiently expressed in the medial sclerotome and the stability of β-galactosidase activity can be used to label the cells. We also introduced the *Uncx4.1-LacZ* transgene into the *Mesp2* KO background and observed the process of vertebrae formation. Our results highlight the importance of IVD/VB differentiation.

Less attention has been paid to IVD development. Several recent studies revealed the roles of Sonic hedgehog signaling in IVD development (Choi and Harfe, 2011; Choi et al., 2012; Maier et al., 2013). Traditional studies suggested that the IVD is derived from the caudal half of the somite, but the sclerotomal origin in the chick embryo appears to be open to debate (Bruggeman et al., 2012; Christ et al., 2007). Some authors have suggested that the IVD and articular processes are derived from somitocoele cells, the internal core of the somite, in avian embryos (Huang et al., 1996; Huang et al., 1994; Mittapalli et al., 2005). However, the relationship between resegmentation and IVD development has not been elucidated in mammalian embryos. To obtain insight into this issue, we took advantage of mouse mutants with defects in the rostro-caudal patterning of the somites. We compared the expression patterns of several IVD (fibromodulin (Smits and Lefebvre, 2003) and TGFβ3 (Millan et al., 1991)) and articular joint (GDF5 (Settle et al., 2003; Storm and Kingsley, 1996)) markers in *Mesp2* KO embryos with caudalized somites and *Ripply1/2* DKO embryos with rostralized somites.

Pax1 is expressed in the whole sclerotome with strong expression in the caudal half of early stage somites and the IVD of late stage embryos (Wallin et al., 1994). To clarify the relationship between rostro-caudal patterning and the IVD, we examined the expression of *Pax1* and *Uncx4.1* at different stages of vertebral column formation in wild-type, *Mesp2* KO, and *Ripply1/2* DKO embryos. Our results show that a periodic pattern of *Pax1* expression correlates with IVD/VB patterning. Taken together, these results imply that differentiation of the IVD and VB is another critical aspect of vertebral column patterning that is intrinsically independent of the rostro-caudal patterning, and that the segmentation of somites and rostro-caudal patterning is not a prerequisite for a metameric pattern, but for regularity and spatial organization of the IVD and VB.

Material and methods

Animals

To generate the *Uncx4.1-LacZ* transgenic mouse, a BAC library was screened for a clone including the *Uncx4.1* locus. We digested the BAC clone with BamHI and co-injected all fragments with the hsp-LacZ-pA cassette into eggs from C57Bl/6; SJL F1 hybrid intercrosses. Microinjection into eggs and oviduct transfer to foster females was performed using standard procedures. Offspring were screened by PCR and X-gal staining of their embryos for β-galactosidase activity in the caudal halves of the somites.

Mesp2$^{+/neo}$ and *Mesp2*$^{+/L}$ mice were maintained in NIHS and represented the most severe *Mesp2* alleles. In *Mesp2*$^{neo/neo}$ and *Mesp2*$^{L/L}$ embryos, compensation by *Mesp1* is suppressed so that no somite boundary is formed and the somitic mesoderm is caudalized (Morimoto et al., 2006; Takahashi et al., 2007b). For observation of β-galactosidase activity, *Uncx4.1-LacZ tg+/–Mesp2*$^{+/neo}$ mice were crossed with *Mesp2*$^{+/neo}$ mice to obtain *Uncx4.1-LacZ tg+/–Mesp2*$^{neo/neo}$ embryos.

Ripply1 and *Ripply2* KO mice were maintained by ST's lab, and *Ripply1–/–Ripply2–/–*embryos were obtained by crossing *Ripply1–/–Ripply2+/–*pairs (Takahashi et al., 2010). Primers for genotyping PCR were provided in previous reports.

X-gal staining of whole mount embryos and frozen sections

E9.5 to E11.5 embryos were stained in whole mounts and subjected to paraffin sectioning. Embryos were fixed in LacZ fix solution at 4 °C for 15 min, washed three times in PBS, and stained in X-gal solution. After post-fixation in 4% paraformaldehyde/PBS