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Growth differentiation factor 11 signaling controls retinoic acid activity for axial vertebral development

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ABSTRACT

Mice deficient in growth differentiation factor 11 (GDF11) signaling display anterior transformation of axial vertebrae and truncation of caudal vertebrae. However, the *in vivo* molecular mechanisms by which GDF11 signaling regulates the development of the vertebral column have yet to be determined. We found that *Gdf11* and *Acvr2b* mutants are sensitive to exogenous RA treatment on vertebral specification and caudal vertebral development. We show that diminished expression of Cyp26a1, a retinoic acid inactivating enzyme, and concomitant elevation of retinoic acid activity in the caudal region of $Gdf11^{-/-}$ embryos may account for this phenomenon. Reduced expression or function of Cyp26a1 enhanced anterior transformation of axial vertebrae in wild-type and *Acvr2b* mutants. Furthermore, a pan retinoic acid receptor antagonist (AGN193109) could lessen the anterior transformation phenotype and rescue the tail truncation phenotype of $Gdf11^{-/-}$ mice. Taken together, these results suggest that GDF11 signaling regulates development of caudal vertebrae and is involved in specification of axial vertebrae in part by maintaining Cyp26a1 expression, which represses retinoic acid activity in the caudal region of embryos during the somitogenesis stage.

Introduction

The vertebral column consists of vertebrae, which protect the spinal cord and provide articulation for movement. Depending on the position and morphological characteristics along the anteroposterior (AP) axis, vertebrae are grouped into cervical, thoracic, lumbar, sacral and caudal vertebrae. The thoracic vertebrae are characterized by the attachment of ribs, and the sacral vertebrae are characterized by the formation of the sacrum. In mammals, from whales to giraffes, the number of cervical vertebrae is invariably seven except for a few species. The number of vertebrae in thoracic, lumbar and sacral regions in mammals is almost invariable within a species but considerably varies between species. For instance, mice have seven cervical (C), thirteen thoracic (T), six lumbar (L), four sacral (S), and over 20 caudal vertebrae, as represented by the C7T13L6S4 vertebral pattern, whereas the human, chimp and horse vertebrae display C7T12L5S5, C7T14L3S5 and C7T18L6S5 patterns, respectively. The vertebral pattern represents the hallmark of the metameric body plan along the AP axis that provides spatial cues for the development of the diaphragm and segmental structures such as the axial muscles, intercostal blood vessels, and projections of spinal nerve systems.

Each vertebra is formed from two adjacent pairs of somites, which also form occipital bones and ribs (Saga and Takeda, 2001). Nascent somites are added to the last segmented somite at a relatively constant rate (about 2 hours in mice) from the presomitic mesoderm (PSM) region, while new mesoderm is concomitantly added at the posterior end of PSM from the tail bud. The manner in which somites acquire their positional information along the AP axis to exhibit their distinctive morphological characteristics has been studied extensively (Baker et al., 2006; Gregg, 2007; Saga and Takeda, 2001). Transplantation experiments in chickens have shown that vertebral specification is established in the PSM region before the segmental plates bud off the PSM and develop into structurally identifiable nascent somites (Nowicki and Burke, 2000). Ample comparative and genetic studies have shown that a specific array of *Hox* genes (a Hox code) is crucial for the specification of a vertebra (Wellik, 2007). Among *Hox* genes, *Hox10* and *Hox11* paralogous genes have been shown to play a role in suppressing rib

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attachments to lumbar and sacral vertebrae and in the formation of the sacrum, respectively (Carapuco et al., 2005; Wellik and Capecchi, 2003). Activities of these *Hox* genes in the PSM, but not in somites, are sufficient for global vertebral patterning (Carapuco et al., 2005). However, the molecular mechanism by which a segmental plate acquires a specific Hox code is poorly understood.

Studies with gain- or loss-of-function mutations in mice have shown that a number of genes are involved in the regulation of multiple *Hox* genes and thereby affect vertebral patterning (Mallo et al., 2009). These genes include the *CDX* family transcription factors (van den Akker et al., 2002), Polycomb group global gene regulators (Akasaka et al., 2001; Core et al., 1997), proteins involved in retinoic acid (RA) synthesis, metabolism, and signaling (Abu-Abed et al., 2001, 2003; Allan et al., 2001; Kessel, 1992; Kessel and Gruss, 1991; Sakai et al., 2001), and proteins involved in GDF11 signaling (Andersson et al., 2006; McPherron et al., 1999; Oh and Li, 1997; Szumska et al., 2008). In this paper we focus on the interactions between RA metabolism and GDF11, two signaling systems involved in vertebral patterning.

Exogenous administration of RA to pregnant females at 8.5 days post coitum (dpc) induces the posterior shift of the Hox code and anterior transformation of vertebrae, resulting in C7/T14/L6 or C7/T15/L5 patterns (Kessel and Gruss, 1991). Homeostasis of RA activity in the caudal region of embryo is essential for the vertebral patterning and the development of caudal vertebrae. In normal mice, RA is inactivated in the caudal region by a cytochrome P450 enzyme, CYP26A1, which catabolizes RA to 4-hydroxy RA (White et al., 1996; Pearlmann, 2002). Repression of RA is essential for the expression of a number of genes, such as *Wnt3a*, *Fgf8* and *bracyury*, in the tail bud region (Abu-Abed et al., 2001, 2003; Sakai et al., 2001). Mice deficient in *Cyp26a1* exhibit markedly elevated RA activity in the tail bud, homeotic transformation of vertebrae, and caudal agenesis (Abu-Abed et al., 2001, 2003; Sakai et al., 2001). Moreover, RA receptor deficiency can rescue the axial vertebral defects of *Cyp26a1*-null mice, demonstrating that elevated RA in the caudal region is the cause of the vertebral defects in *Cyp26a1*$^{-/-}$ mice (Abu-Abed et al., 2003).

GDF11 is a member of the transforming growth factor-β (TGF-β) superfamily and is involved in axial vertebral patterning and development of the palate, kidney, and pancreas (Dichmann et al., 2006; Esquela and Lee, 2003; McPherron et al., 1999). The active form of TGF-β superfamily proteins is generated by proteolytic cleavage of the precursor protein. Recent studies have shown that proprotein convertase PCSK5 (PC5/6) is necessary for the activation of Gdf11 (Essalmani et al., 2008; Seidah et al., 2008; Szumska et al., 2008). The TGF-β family signal is transduced through interactions with heteromeric complexes of type II and type I receptors (Massague, 1998). Activin type II receptors (ACVR2A and ACVR2B) and the TGF-β type I receptor (ALK5; TGFBR1) have been shown to mediate the GDF11 signal for vertebral specification (Andersson et al., 2006; Oh and Li, 1997; Oh et al., 2002). SMAD2 and SMAD3 are known cytoplasmic targets of ACVR2/2B and ALK5 (Massague, 1998; Oh et al., 2002).

Gdf11$^{-/-}$ mice exhibit anterior transformations of the axial skeleton, resulting in an increased number of thoracic and lumbar vertebrae (C7/T18/L8) and truncation of caudal vertebrae (McPherron et al., 1999). GDF11 has functional redundancy with GDF8 (myostation; Mstn) in patterning and development of the axial skeleton (McPherron et al., 2009): most *Gdf11*$^{-/-}$;*Mstn*$^{-/-}$ mice have an increase in severity of anterior transformation (mostly 20 thoracic vertebrae) and tail truncation (up to sacral vertebrae), as compared to *Gdf11*$^{-/-}$ mice. The vertebral transformation defects of *Gdf11*$^{-/-}$;*Mstn*$^{-/-}$ mice represent the most remarkable phenotype among all known vertebral patterning defects of multiple mutant mice in terms of the extent of the transformation. The closest phenotypic resemblance is found in mice with triple *Hox* gene deletion (Wellik and Capecchi, 2003; McIntyre et al., 2007), suggesting that expressions of multiple *Hox* genes are affected in *Gdf11*$^{-/-}$ mice. Consistent with this, it has been demonstrated that the expression boundaries of multiple *Hox* genes are shifted posteriorly in *Acvr2b*$^{-/-}$, *Gdf11*$^{-/-}$, and *Pcsk5*$^{-/-}$ mice (Essalmani et al., 2008; McPherron et al., 1999; Oh and Li, 1997; Szumska et al., 2008). However, the mechanism by which Gdf11 signaling controls multiple *Hox* genes for axial vertebral patterning remains unknown.

In this paper, we present data suggesting that GDF11 signaling is an important determinant for the RA gradient along the AP axis by regulating CYP26A1 expression in the tail bud region for proper vertebral specification and tail development.

Materials and methods

Mouse strains

All mouse strains used in this study are listed as follows: *Gdf11*-, *Acvr2a*-, *Acvr2b*-, *Cyp26a1*-knockout mice, and *RARE-LacZ* transgenic mice (McPherron et al., 1999; Oh and Li, 1997; Rossant et al., 1991; Sakai et al., 2001; Song et al., 1999; Yang et al., 1999). Mice were maintained under standard specific-pathogen-free conditions and all animal procedures performed were reviewed and approved by the University of Florida and Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee.

Mouse mating schemes

For monitoring the *in vivo* RA activity, *Gdf11*$^{+/-}$;*RARE-lacZ(+)* and *Acvr2b*$^{+/-}$;*RARE-lacZ(+)* males were intercrossed with *Gdf11*$^{+/-}$ and *Acvr2b*$^{+/-}$ females, respectively. Embryos were collected at E8.5 and E10.5 for X-gal staining. For genetic interaction between *Acvr2b* and *Cyp26a1*, *Acvr2b*$^{-/-}$ males were intercrossed with *Acvr2b*$^{+/-}$; *Cyp26a1*$^{+/-}$ females, and the vertebral patterns of *Acvr2b*$^{-/-}$; *Cyp26a1*$^{+/-}$ newborn pups were compared with those of *Acvr2b*$^{-/-}$ and *Acvr2b*$^{+/-}$;*Cyp26a1*$^{+/-}$ pups. For the RA sensitivity study, *Acvr2b*$^{-/-}$ males were intercrossed with *Acvr2b*$^{+/-}$;*Acvr2a*$^{+/-}$ females. RA was administered to dams at 8.5 dpc as described below.

Administration of R115866, retinoic acid, and AGN193109

10 mM R115866 (Johnson & Johnson Co.) stock was made in DMSO and the aliquots were stored in −20 °C freezer. The stock solution was diluted in PEG200 just prior to use and administered to pregnant dams at 8.5 dpc via oral gavage needles. All-*trans* RA (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO at 25 mg/ml and stored at −20 °C in the dark. The RA stock solution was subjected to a 10-fold dilution in sesame oil and orally administrated to the pregnant mice at 8.5 dpc at a final concentration of 10 mg/kg of body weight. AGN193109 (Allergan Inc., Irvine, CA) was dissolved in DMSO at 1 mg/ml and stored at −20 °C freezer. The stock solution was diluted in corn oil just before use and administrated to pregnant dams at 8.5 and/or 9.5 dpc through oral gavage needles at a final concentration of 2 mg/kg of body weight.

Skeleton preparation

E17.5, E18.5, or newborn pups were subjected to skeleton preparations as previously described (Lee et al., 2006). Mice were eviscerated and left in water overnight with gentle shaking. After further removal of skin, fat, muscle, and glands, the sample was fixed in 95% ethanol for 2 to 5 days. The sample was then stained overnight in Alcian blue 8GX staining solution (0.15 mg/ml in 80% ethanol and 20% glacial acetic acid), and rinsed with 95% ethanol. After the tissue debris was cleared in 2% KOH solution for 3 hr, the skeleton was stained with 0.005% alizarin red S in 2% KOH for 3 h. The stained skeleton was rinsed with 2% KOH and kept in 50% glycerol/PBS.