Growth differentiation factor 11 locally controls anterior–posterior patterning of the axial skeleton

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Abstract
Growth and differentiation factor 11 (GDF11) is a transforming growth factor β family member that has been identified as the central player of anterior–posterior (A–P) axial skeletal patterning. Mice homozygous for Gdf11 deletion exhibit severe anterior homeotic transformations of the vertebrae and craniofacial defects. During early embryogenesis, Gdf11 is expressed predominantly in the primitive streak and tail bud regions, where new mesodermal cells arise. On the basis of this expression pattern of Gdf11 and the phenotype of Gdf11 mutant mice, it has been suggested that GDF11 acts to specify positional identity along the A–P axis by either local changes in levels of signaling as development proceeds or by acting as a morphogen. To further investigate the mechanism of action of GDF11 in the vertebral specification, we used a Cdx2-Cre transgene to generate mosaic mice in which Gdf11 expression is removed in posterior regions including the tail bud, but not in anterior regions. The skeletal analysis revealed that these mosaic mice display patterning defects limited to posterior regions where Gdf11 expression is deficient, whereas displaying normal skeletal phenotype in anterior regions where Gdf11 is normally expressed. Specifically, the mosaic mice exhibited seven true ribs, a pattern observed in wild-type (wt) mice (vs. 10 true ribs in Gdf11−/− mice), in the anterior axis and nine lumbar vertebrae, a pattern observed in Gdf11 null mice (vs. six lumbar vertebrae in wt mice), in the posterior axis. Our findings suggest that GDF11, rather than globally acting as a morphogen secreted from the tail bud, locally regulates axial vertebral patterning.

KEYWORDS
Cdx2-Cre, GDF11, skeletal patterning, tail bud

1 | INTRODUCTION

Vertebrates, despite varying remarkably in body shape and size, share highly conserved developmental mechanisms regulating body segment positioning from head to tail (Mallo, 2018). During the process of skeletal patterning along the anterior–posterior (A–P) axis, coordinated cell signaling events induce sequential addition of new tissue from progenitors at the posterior end of an embryo, eventually forming the...
axial skeleton composed of the skull, vertebral column, and thoracic cage (Wellik, 2007; Wilson, Olivera-Martinez, & Storey, 2009; Wyneersch et al., 2016). While vertebrae and ribs develop from adjacent pairs of somites, their positional information is determined in the presomitic mesoderm region before the actual formation of nascent somites (Carapuco, Novoa, Bobola, & Mallo, 2005; Kiency, Mauger, & Sengel, 1972; Nowicki & Burke, 2000; Saga & Takeda, 2001). Such positional information is thought to be provided by morphogens, or signaling molecules secreted from the signaling center, which acts at long range in a concentration-dependent manner to control specific combinatorial expressions of Hox genes, ensuring proper body patterning of developing embryos (Schilling, Nie, & Lander, 2012; Tickle, Summerbell, & Wolpert, 1975).

Growth and differentiation factor 11 (GDF11), a vertebrae-conserved transforming growth factor β (TGF-β) family member also known as bone morphogenetic protein 11 (BMP11), has been identified as the key molecule that determines positional identity of the axial skeleton by modulating Hox gene expression (Gamer et al., 1999; Jurberg, Aires, Varela-Lasheras, Novoa, & Mallo, 2013; Matsubara et al., 2017; McPherron, Lawler, & Lee, 1999). Mice homozygous for Gdf11 deletion are perinatally lethal and display patterning defects characterized by anteriorly directed transformations of the vertebral column, leading to the extended trunk and shortened tail. Unlike normal mice that represent 13 thoracic, six lumbar vertebrae, and seven true (vertebrosternal) ribs, that represent 13 thoracic, six lumbar vertebrae, and seven true ribs (McPherron et al., 1999). Conversely, mice expressed in posterior regions by E8.5 (Hinoi et al., 2007; Silberg, Suh, & Traber, 2000), to target recombination specifically in the caudal region of embryos carrying a floxed Gdf11 allele. Here, we demonstrate that mosaic mice lacking Gdf11 expression in posterior regions display abnormal skeletal patterning limited to the regions where Gdf11 gene is removed, suggesting that GDF11 does not act globally as a morphogen secreted from the tail bud, but acts locally to control axial skeletal patterning.

2 | MATERIALS AND METHODS

2.1 | Mice

All animal studies were approved by the Institutional Animal Care and Use Committees at Seoul National University. Generation of Gdf11 conditional knockout mice has been previously described (McPherron, Huynh, & Lee, 2009). To analyze the effect of Cdx2-Cre on Gdf11fl/fl mice, Cdx2-Cre transgenic male mice (Stock No. 009350), purchased from the Jackson Laboratory (Bar Harbor, ME), were first mated with Gdf11fl/fl female mice. Subsequently, Cdx2-Cre; Gdf11fl/fl male mice were mated with Gdf11fl/fl females to obtain Cdx2-Cre; Gdf11fl/fl mice for analysis. Gdf11 conditional knockout mice were also crossed to Ella-Cre transgenic female mice to generate mice heterozygous for the deletion allele (Gdf11fl/−), and Gdf11fl/− mice were intercrossed to generate Gdf11−/− mice. Because both Cdx2-Cre; Gdf11fl/fl and Gdf11−/− mice were perinatal lethal, the skeletal analysis was performed at P0. IgS-creGdf11floxGfp/+ mice were kindly provided by Max A. Tischfield and Jeremy Nathans. All mice were maintained on a C57BL/6 background.

2.2 | Whole-mount in situ hybridization

E9.5 embryos were prepared and stained using digoxigenin-labeled Gdf11 probes as previously described (McPherron et al., 1999). Briefly, embryos were hybridized at 65°C overnight, washed, and incubated with 1:4000 dilution of alkaline phosphatase-conjugated antibody (Sigma) at 4°C overnight. The color reaction was performed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Sigma).

2.3 | Skeletal staining

Newborn mice were prepared and stained using Alcian blue/Alizarin red solution as previously mentioned (McPherron et al., 1999). In short, mice were skinned, eviscerated, fixed, and dehydrated in ethanol and acetone. Subsequently, mice were stained for 36 hr at 37°C with a solution containing 0.003% Alizarin red (Sigma), 0.0045% Alcian blue (Sigma), and 10% acetic acid in ethanol. Finally, mice were incubated in 1% potassium hydroxide for 4 days and gradually transferred to glycerol. Photos were taken using a stereomicroscope (Zeiss) and AxiosVision software (Zeiss).

2.4 | Micro-computed tomography

Newborn mouse skulls and vertebrae were scanned using inspeXio SMX-90CT (Shimadzu) with pixel sizes of 25 and 45 μm, respectively at 90 kV and 110 μA. Images were reconstructed and
displayed using a manufacturer-provided software TRI/3D-BON (RATOC System).

2.5 | Fluorescence microscopy

Green fluorescent protein (GFP)-positive regions of E9.5 Cdx2-Cre; Gdf11\textsuperscript{flox/flox}; Igs1\textsuperscript{CKI-mitoGFP+} embryos and newborn Cdx2-Cre; Gdf11\textsuperscript{flox/flox}; Igs1\textsuperscript{CKI-mitoGFP+} mice were visualized using Axio Observer Z1 (Zeiss) and Zen software (Zeiss).

3 | RESULTS

3.1 | Gdf11 expression is eliminated in caudal regions of the mosaic embryos

Using a Cdx2-Cre transgene in conjunction with a floxed Gdf11 allele, we produced Cdx2-Cre; Gdf11\textsuperscript{flox/flox} mosaic mice in which Gdf11 expression is eliminated in posterior but not in anterior regions. To confirm the deletion of Gdf11 expression specific to Cdx2-Cre expressing regions, we performed whole-mount in situ hybridization for Gdf11 expression in E9.5 embryos. In line with previous reports (McPherron et al., 1999; Nakashima et al., 1999), Gdf11 expression was detected predominantly in the tail bud, mildly along with the dorsal tissues, and craniofacial regions in wild-type (wt) embryos. However, in Cdx2-Cre; Gdf11\textsuperscript{flox/flox} embryos, Gdf11 expression was absent specifically in the tail bud and posterior dorsal regions (Figure 1a). To visualize Cdx2-Cre transgene action, we utilized Igs1\textsuperscript{CKI-mitoGFP+} conditional knock-in mice to induce Cdx2-Cre-mediated recombination of a floxed stop cassette to enable GFP expression exclusively in mitochondria of Cdx2-Cre-positive cells (Agarwal et al., 2017). As expected, fluorescence imaging of Cdx2-Cre; Gdf11\textsuperscript{flox/flox}; Igs1\textsuperscript{CKI-mitoGFP+} embryos at E9.5 revealed GFP expression only in posterior regions corresponding to those lacking Gdf11 expression in Cdx2-Cre; Gdf11\textsuperscript{flox/flox} embryos (Figure 1b). Newborn Cdx2-Cre; Gdf11\textsuperscript{flox/flox} and Gdf11\textsuperscript{−/−} mice displayed indistinguishable outward appearance, both displaying extended torso and truncated tail (Figure 1c). Both mice also exhibited perinatal lethality although Cdx2-Cre; Gdf11\textsuperscript{flox/flox} mice tended to live slightly longer. Analysis of GFP expression in newborn Cdx2-Cre; Gdf11\textsuperscript{flox/flox}, Igs1\textsuperscript{CKI-mitoGFP+} mice once again confirmed that Cdx2-Cre action is limited to posterior tissues of the conditional knockout mice (Figure 1d).

3.2 | Mosaic mice display skeletal patterning defects limited to posterior regions

Normal mice represent the vertebral formula of seven cervical, 13 thoracic, six lumbar, and seven true ribs. However, mutation in Gdf11 results in anteriorly directed homeotic transformations of vertebrae and ribs in a dose-dependent manner as Gdf11\textsuperscript{−/−} mice display 18 thoracic, nine lumbar, and 10 true ribs, and Gdf11\textsuperscript{−/−} mice display 14 thoracic, six lumbar, and eight true ribs (Lee & Lee, 2017). Mosaic mice displayed 18 thoracic, nine lumbar, and 10 true ribs (Figure 1c). Both mice also exhibited perinatal lethality although Cdx2-Cre; Gdf11\textsuperscript{flox/flox} mice tended to live slightly longer. Analysis of GFP expression in newborn Cdx2-Cre; Gdf11\textsuperscript{flox/flox}, Igs1\textsuperscript{CKI-mitoGFP+} mice once again confirmed that Cdx2-Cre action is limited to posterior tissues of the conditional knockout mice (Figure 1d).

FIGURE 1 Gdf11 expression is removed in posterior regions of Cdx2-Cre; Gdf11\textsuperscript{flox/flox} mice. (a) Whole-mount in situ hybridization of mouse embryos at E9.5. Gdf11 expression patterns of wt and Cdx2-Cre; Gdf11\textsuperscript{flox/flox} embryos are shown. Dashed line with arrow heads indicates posterior regions that lack Gdf11 expression in a Cdx2-Cre; Gdf11\textsuperscript{flox/flox} embryo. (b) Cells expressing Cdx2-Cre are marked by GFP expression in Cdx2-Cre; Gdf11\textsuperscript{flox/flox}; Igs1\textsuperscript{CKI-mitoGFP+} embryo at E9.5. (c) Newborn wt, Gdf11\textsuperscript{−/−}, and Cdx2-Cre; Gdf11\textsuperscript{flox/flox} pups. Both Gdf11\textsuperscript{−/−} and Cdx2-Cre; Gdf11\textsuperscript{flox/flox} mice display extended torso and truncated tails. (d) Area expressing Cdx2-Cre is labeled by GFP expression in newborn Cdx2-Cre; Gdf11\textsuperscript{flox/flox}, Igs1\textsuperscript{CKI-mitoGFP+} mouse, and displayed laterally and ventrally. GFP, green fluorescent protein; wt, wild-type [Color figure can be viewed at wileyonlinelibrary.com]
Although newborn Cdx2-Cre; Gdf11<sup>flox/flox</sup> mice share identical external appearance with Gdf11<sup>−/−</sup> mice, further examination demonstrated that they exhibit dissimilar skeletal patterns. In detail, Alcian blue/Alizarin red staining and micro-computed tomography (micro-CT) analysis of newborn mice revealed that while both Cdx2-Cre; Gdf11<sup>flox/flox</sup> and Gdf11<sup>−/−</sup> mice display nine lumbar vertebrae, Cdx2-Cre; Gdf11<sup>flox/flox</sup> and Gdf11<sup>−/−</sup> mice display 15 and 18 thoracic vertebrae, respectively (Figures 2a, c and S1 and Table 1), showing milder defects in thoracic vertebrae of the mosaic mice. In anterior regions, however, Cdx2-Cre; Gdf11<sup>flox/flox</sup> mice exhibited normal phenotype, displaying seven true ribs, unlike Gdf11<sup>−/−</sup> mice that expressed 10 true ribs (Figure 2b, c and Table 1). Likewise, both Cdx2-Cre; Gdf11<sup>flox/+</sup> and Gdf11<sup>−/−</sup> mice exhibited 14 thoracic and six lumbar vertebrae (Figure 2a), but only Cdx2-Cre; Gdf11<sup>flox/+</sup> mice represented normal true ribs (Figure 2b). To summarize, in anterior regions where Gdf11 is not targeted, Cdx2-Cre; Gdf11<sup>flox/+</sup> mice display normal skeletal patterns but in posterior regions where Gdf11 is targeted,
Cdx2-Cre; Gdf11−/−, and Cdx2-Cre; Gdf11flox/flox mice present anteriorly directed homeotic transformations, developing nine lumbar vertebrae, similar to what is observed in Gdf11−/− mice (Figures 2c and S1). In addition, fluorescence imaging of newborn Cdx2-Cre; Gdf11flox/flox; IgscCKI-mitoGFP/+ mice demonstrated that patterning defects arise below eighth thoracic vertebra, where Gdf11 deletion is visualized by GFP expression, but not above eighth thoracic vertebra where Gdf11 is expressed normally (Figure 2d), suggesting that locally expressed GDF11, not GDF11 secreted from the tail bud, defines positional identity in the axial skeleton.

3.3 | Gdf11 null mice, but not the mosaic mice, exhibit craniofacial defects

The palate, which separates the oral and nasal cavity, starts to develop around E10.5 in the mouse embryo as the medial nasal processes fuse with maxillary processes to form the primary palate. Subsequently, palatal outgrowths emerge from the maxillary processes, forming the secondary palate, and expand vertically to become palatal shelves, which begin to fuse around E15 (Bush & Jiang, 2012; Funato, Nakamura, & Yanagisawa, 2015). Interference of these events by genetic or environmental factors can lead to the formation of a cleft palate. GDF11 has been shown to play an essential role in normal craniofacial development as Gdf11 is notably expressed in craniofacial regions at E9.5 (Figure 1a) and E10.5 (Nakashima et al., 1999), and Gdf11−/− mice exhibit a cleft palate with high penetrance (Lee & Lee, 2013, 2015; McPherron et al., 1999). Likewise, our micro-CT and Alcian blue/Alizarin red staining analysis revealed that over 60% of newborn Gdf11−/− mice were born with a cleft palate accompanied by the wide spacing between the pterygoid processes. However, no craniofacial defects were observed in Cdx2-Cre; Gdf11flox/flox mice that displayed normal palate formation (Figure 3a,b and Table 1). In fact, normal Gdf11 expression was detected in craniofacial regions of Cdx2-Cre; Gdf11flox/flox mouse embryos (Figure 1a), suggesting that GDF11 locally contributes to proper craniofacial development.

4 | DISCUSSION

Mice deficient in Gdf11 represent one of the most severe axial skeleton patterning defects, marking GDF11 as the pivotal regulator of vertebral skeleton segmentation along the A–P axis. Specifically, GDF11 has been identified as a secreted signaling molecule of the TGF-β family.

| Genotypes of mutant mice | Cdx2-Cre | Gdf11 |
|--------------------------|----------|-------|
|                         | +/-      | +/-   |
| N                       | 10       | 13    |
| Palate                  |          |       |
| Intact                  | 10       | 13    |
| Cleft                   | –        | 6     |
| Anterior tuberculi on no. vertebrae |
| C6                      | 10       | –     |
| C7                      | –        | 1     |
| Attached ribs           |
| 7                       | 10       | –     |
| 8                       | –        | 13    |
| 9                       | –        | –     |
| 10                      | –        | –     |
| 11                      | –        | –     |
| Total no. of thoracic vertebrae |
| 13                      | 10       | –     |
| 14                      | –        | 13    |
| 15                      | –        | –     |
| 16                      | –        | –     |
| 17                      | –        | –     |
| 18                      | –        | 7     |
| Total No. of lumbar vertebrae |
| 5                       | –        | –     |
| 5/6                     | –        | –     |
| 6                       | 10       | 13    |
| 6/7                     | –        | –     |
| 7                       | –        | –     |
| 7/8                     | –        | 6     |
| 8/9                     | –        | –     |
| 9                       | –        | 3     |

Abbreviation: wt, wild-type.
predominantly expressed in the tail bud of a developing embryo. This led to the concept that the tail bud acts as a signaling center to secrete GDF11, which behaves as a morphogen to specify the vertebral formula (McPherron et al., 1999). However, Gdf11 expression is also detected outside the tail bud along the dorsal regions, although less prominently compared with that observed in the tail bud (Figure 1a), and whether GDF11 originated from non-tail bud areas regionally contributes to patterning was not clearly determined. We believed that if the tail bud

**FIGURE 3** Craniofacial defects are observed in Gdf11−/− mice, but not in Cdx2-Cre; Gdf11flox/flox mice. (a) Representative micro-CT images of newborn mouse skulls shown ventrally. Red arrows point to palatine bones, and yellow arrows indicate pterygoid processes. Note that cleft palate is observed only in Gdf11−/− mice. (b) Alcian blue/Alizarin red staining of newborn skulls. Boxed regions are shown at higher magnification. Yellow arrows indicate pterygoid processes. Cleft palate is observed in Gdf11−/− mice, but not in Cdx2-Cre; Gdf11flox/flox mice. micro-CT, micro-computed tomography; wt, wild-type [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 4** Schematic representation of vertebral columns indicating that locally expressed GDF11, not GDF11 secreted from the tail bud, controls axial skeletal patterning. Cdx2-Cre; Gdf11flox/flox mice display an extended number of posterior vertebrae where GDF11 expression is removed, but normal patterning of anterior vertebrae. Cervical (orange)/thoracic (purple)/lumbar (sky blue) vertebrae, anterior tuberculi (small blue dots), sternums (red curves), and ribs (blue lines) are color-coded as indicated. Gray-dashed lines indicate normal vertebral positions: Six for the anterior tuberculum, 20 for the final thoracic vertebra, and 26 for the last lumbar vertebra. The green-dashed line represents the upper limit of Cdx2-Cre expression. GDF11, growth and differentiation factor 11; wt, wild-type [Color figure can be viewed at wileyonlinelibrary.com]
truly is the major signaling center and source for GDF11, specific deletion of Gdf11 in the tail bud would yield identical skeletal patterns to global deletion of Gdf11. To selectively remove Gdf11 expression in the tail bud, we utilized a genetic approach, incorporating a Cdx2-Cre transgene to target recombination exclusively in the caudal region of mouse embryos harboring a floxed Gdf11 allele. The initial screening revealed no differences in external appearance between newborn Cdx2-Cre; Gdf11\[^{lox/lox}\] and Gdf11\[^{−/−}\] mice, both displaying elongated trunk and shortened tail. However, closer examination uncovered disparity between their skeletal patterns; in skeletons below eighth thoracic vertebra where floxed Gdf11 alleles are excised by Cdx2-Cre recombinase, Cdx2-Cre; Gdf11\[^{lox/lox}\] mice exhibited anteriorly directed homeotic transformations equal to those observed in Gdf11\[^{−/−}\] mice, but in skeletons above eighth thoracic vertebra where Gdf11 is normally expressed, Cdx2-Cre; Gdf11\[^{lox/lox}\] mice displayed normal skeletal patterns. In detail, characteristics of the anterior skeletons including the number of true ribs, cervical position of anterior tuberculum, and craniofacial development were all normal in Cdx2-Cre; Gdf11\[^{lox/lox}\] and Cdx2-Cre; Gdf11\[^{lox/−}\] mice as opposed to Gdf11\[^{−/−}\] and Gdf11\[^{−/−}\] mice (Figures 2A, 4, and S1). Because Cdx2-Cre; Gdf11\[^{lox/lox}\] mice showed skeletal defects limited to posterior regions where Gdf11 expression is eliminated, our data suggest that GDF11, rather than globally acting as a morphogen secreted from the tail bud, locally stimulates the expression of cytochrome P450 enzyme, CYP26A1, through ACVR2 signaling (Lee et al., 2010). Consistent with this, accumulating evidence suggests that GDF11 stimulates the expression of posterior 5′ Hox genes, Hox9 to Hox13 paralogs, whereas suppressing the expression of anterior 3′ Hox genes (Aires et al., 2016, 2019; Matsubara et al., 2017), although whether through ACVR2 signaling and inhibition of RA, or through BMP signaling is still unclear.

In the present study, we have shown that GDF11 locally regulates axial skeletal patterning rather than globally acting as a morphogen secreted from the tail bud. During embryogenesis, Gdf11 expression level is the highest in the posterior end of the primitive streak and tail bud and gradually fades anteriorly (Figure 1A; McPheron et al., 1999; Nakashima et al., 1999). From our data, it seems likely that high expression of GDF11 in posterior ends of the embryo locally induces strong activation of posterior 5′ Hox and inhibition of 3′ Hox genes, coordinating the formation of posterior vertebrae, whereas mild expression of GDF11 locally regulates the positioning of relatively more anterior vertebrae through moderate activation of both posterior 5′ Hox and anterior 3′ Hox genes. It might be interesting to further clarify the functional relationship between local GDF11 signaling and Hox gene regulation during vertebral patterning in future studies.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Y.-S.L. conceived and designed the study; J.S. performed most of the experiments with assistance from J.-H.E. and N.-K.K.; S.-J.L. provided most of the genetically engineered mice; J.S., K.M.W., J.-H.B., H.-M.R., and Y.-S.L. analyzed data; and J.S., S.-J.L., and Y.-S.L. wrote the paper.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available within the article.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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