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Cadherin-6B is required for the generation of Islet-1-expressing dorsal interneurons

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ABSTRACT

Cadherin-6B induces bone morphogenetic protein (BMP) signaling to promote the epithelial mesenchymal transition (EMT) in the neural crest. We have previously found that knockdown of Cadherin-6B inhibits both BMP signaling and the emigration of the early pre-migratory neural crest cells from the dorsal neural tube. In this study, we found that inhibition of BMP signaling in the neural tube, mediated by the ectopic expression of Smad-6 or Noggin, decreased the size of the Islet-1-positive dorsal cell population. Knockdown or loss of function of Cadherin-6B suppressed the generation of Islet-1-expressing cells in the dorsal neural tube, but not the Lim-1/2 positive dorsal cell population. Our results thus indicate that Cadherin-6B is necessary for the generation of Islet-1-positive dorsal interneurons, as well as the initiation of pre-migratory neural crest cell emigration.

1. Introduction

Distinct cell populations are generated from three different progenitor domains in the dorsal neural tube: the roof plate, the neural crest, and the dorsal neural progenitor domain [1]. The generation of these domains is spatially and temporally regulated by various extrinsic and intrinsic signals [1,2]. More importantly, a subpopulation of progenitor cells in the early dorsal neural tube is able to differentiate into all three progenitor populations [2–4]. However, dorsal neural progenitors mostly differentiate into early dorsal interneurons and can be divided into six classes, dorsal interneurons 1–6 (dI1-dI6). The specification of each class is regulated by the expression of ligands for various signaling pathways in the roof plate, such as BMP [5] and Wingless-type MMTV integration site family members (Wnt) [1,6–9].

BMPs are among the most important extrinsic factors controlling the specification of dorsal interneurons [2,10], as well as the generation and emigration of the neural crest cells [11,12]. BMP signaling is transduced through canonical and non-canonical pathways. In canonical transduction of BMP signals, ligands induce the formation of a heterodimer composed of a type 1 and a type 2 receptor. Type 2 receptors activate type 1 receptors through phosphorylation. Then, the activated type 1 receptor phosphorylates Smad-1, 5, 8 and these phosphorylated Smad proteins enter the nucleus after binding with Smad-4 in order to regulate the transcription of BMP target genes. Non-canonical transduction of BMP signals is dependent on the physical/functional interactions of BMP receptors with a variety of proteins, including c-Jun N-terminal kinases, Tribbles-like protein-3, Scr, Lim kinase-1, and Cadherin-6B [13–17].

Cadherins are adhesion molecules which form cell–cell adherence junctions and also play important roles in signal transduction [18]. Cadherin-6B is expressed in the dorsal neural tube [12,19] and is required for the epithelial mesenchymal transition (EMT) in the trunk neural crest [20,21]. Importantly, Cadherin-6B can intrinsically activate BMP signaling independently of BMP ligands through the regulation of BMP receptors [17]. Therefore, it is possible that Cadherin-6B regulates both the EMT in the neural crest and the generation of the other BMP-regulated cell populations, such as the dorsal interneurons. Thus, we sought to determine if there are roles for Cadherin-6B dependent BMP signaling in dorsal interneuron development.

2. Materials and methods

2.1. Chicken embryos

We incubated fertilized chicken (*Gallus gallus*) eggs (Charles River Laboratories, North commons, Conn., USA) at 38 °C in a

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humidified incubator (G. Q. F. Manufacturing, Savannah, Ga., USA). Embryos were staged according to the number of somite pairs.

2.2. Electroporation and DNA constructs

DNA constructs were prepared and electroporation was performed as previously described [20]. Thirteen to 15 somite pair stage embryos were electroporated with the following DNA constructs: pCIG containing an IRES-nuclear green fluorescence protein (GFP) expression construct, pCIG-GPI-Cadherin-6B [Park and Gumbiner, 2010], pCAGGS-Noggin-CD4-IRES-GFP, pCAβ-chick-Smad6-IRES-GFP, and pSilencer-2.0-U6-Cadherin-6B [20]. The electroporated embryos were incubated for 40–48 h. In Cadherin-6B knockdown experiments, a mixture of both pCIG and pSilencer-2.0-U6 Cadherin-6B was electroporated.

2.3. Tissue preparation and immunohistochemistry

Tissue preparation and immunohistochemistry were carried out as previously described [20]. Briefly, the trunk region of fixed embryos (between 20 and 24 somite pair) was dissected out, cryopreserved and frozen, and then cryo-sectioned at 12 μm. Immunohistochemistry was performed with primary antibodies against the following proteins: Islet-1 [Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA; 1:100 dilution], Lim-1/2 (DSHB; 1:100 dilution), AP-2 (DSHB; 1:100 dilution), HNK-1 (hybridoma-producing primary monoclonal antibody supernatant from the American Type Culture Collection, Manassas, VA, USA; 1:50 dilution), and Laminin (Sigma, MO, St. Louis, USA; 1:500 dilution). The following secondary antibodies (AlexaFluor; Invitrogen, Carlsbad, Calif., USA) were used at a 1:1000 dilution: anti-rabbit IgG-Alexa 546, anti-rabbit IgG-Alexa 633, anti-mouse IgG-Alexa 546, anti-mouse IgG2b-Alexa 546, anti-mouse IgG1-Alexa 633, and anti-mouse IgM-Alexa 546. When necessary, TO-PRO (Invitrogen) was used for nucleus staining. Images were collected with a Nikon Eclipse TE2000 confocal microscope.

2.4. Statistics

Student's *t*-tests were carried out to determine statistical significance.

3. Results

3.1. Cadherin-6B is necessary for emigration of neural crest cells from the neural tube

We first confirmed that Cadherin-6B inhibits the emigration of the neural crest cells (Fig. 1). We electroporated either an empty vector construct containing the GFP coding sequence linked with an IRES sequence (pCIG) or a mixture of pCIG and *cadherin-6B* shRNA (shCad6B) into stage 13–15 chick embryos. GFP expression was used to identify electroporated cells. We observed a decreased number of emigrating GFP-positive cells in the Cadherin-6B knockdown embryos compared to control (pCIG) (Fig. 1A, B). We also tested a dominant-negative *cadherin-6B* construct, in which the extracellular domain of Cadherin-6B is linked with a glycosyl-phosphatidylinositol (GPI) anchor, in order to confirm the shRNA experiments and assess the effect of functional knockdown of Cadherin-6B on the emigration of neural crest cells [20]. We consistently observed that significantly fewer dominant-negative Cadherin-6B (GPI-Cad6B)-expressing GFP positive cells emigrated from the neural crest as compared to control (pCIG) (Fig. 1C, D). Taken together, these results demonstrate that both knockdown of *cadherin-6B* transcripts and loss of Cadherin-6B function inhibited emigration from the trunk neural crest. To assess

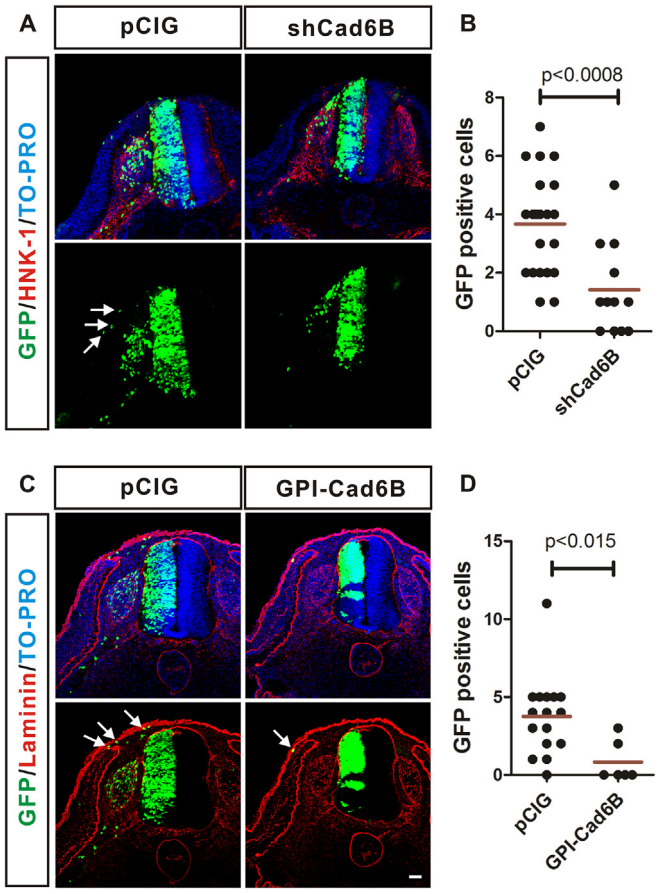

Fig. 1. Loss of Cadherin-6B diminished emigration of truck neural crest cells. (A–B) Embryos (13-15 somite pair stage) were electroporated with either a control vector (pCIG, n = 3 embryos) or a mixture of pCIG and the shRNA-expressing vector targeting *cadherin-6B* (shCad6B, n = 3 embryos). Immunostaining of HNK-1 (red) marks neural crest cells emigrating from the neural tube. GFP positive cells which migrate subectodermally were quantitatively analyzed (B). Red lines indicate mean values and p value was calculated with Student's t-test. Total 21 sections of the control embryos and total 12 sections of Cadherin-6B knockdown embryos were analyzed. (C–D) A control vector (pCIG, n = 3 embryos) or a construct expressing a dominant negative form of *cadherin-6B* (GPI-Cad6B, n = 4 embryos) was electroporated into the neural tube. Electroporated cell were labeled by the expression of GFP from an IRES-GFP in each construct. Immunostaining of Laminin (red) delineates the basement membrane of the neural tube. GFP positive cells which migrate subectodermally were quantitatively analyzed (D). Red lines indicate mean values and p value was calculated with Student's t-test. Total 16 sections or 6 sections of pCIG experimental group or GPI-Cad6B were analyzed, respectively. Nuclei were stained with TO-PRO (blue, A-B). White arrows denote the migrating GFP positive cells. Scale bar = 25 μm.

whether the diminished emigration from the trunk neural crest coincides with the accumulation of neural crest cells in the dorsal neural tube, we performed immunohistochemistry for AP-2, which is expressed in both pre-migratory and migratory neural crest cells. To our surprise, loss of Cadherin-6B did not affect the population size of AP-2-expressing cells in the dorsal-lateral neural tube and AP-2 positive pre-migratory neural crest cells did not accumulate inside of the dorsal neural tube after Cadherin-6B knockdown (Fig. S1). These results suggest that Cadherin-6B-deficient neural crest cells that were unable to migrate outside of the neural tube may change their cellular fate inside of the neural tube.

3.2. Cadherin-6B regulates the generation of dorsal interneurons

BMP signaling is essential for both the EMT in the avian neural crest [12,22] and the patterning/generation of dorsal interneurons

[5,23]. It has been previously reported that Cadherin-6B stimulates BMP signaling [17,20] and knockdown of Cadherin-6B partially blocked the activation of BMP signaling mediated by binding of BMP-4 ligand (data now shown). These findings raised the possibility that Cadherin-6B might regulate the generation of the dorsal interneurons upstream of the BMP pathway. To address this hypothesis, we firstly determined whether the inhibition of BMP signaling affected the generation of dorsal interneurons in the chick neural tube. To test this, we first inhibited BMP signaling by electroporating constructs for Noggin (an antagonist of BMP ligand) or Smad-6 (an inhibitory Smad) and observed a diminution in the number of Islet-1-positive dorsal interneurons (dI3) (Fig. 2). Interestingly, Cadherin-6B deficiency induced by either shRNA or dominant negative expression construct also decreased the number of Islet-1-positive dorsal interneurons (Fig. 3). The decline in the Islet-1-positive dorsal neurons was more significant with dominant negative Cadherin-6B than shRNA-mediated knockdown of Cadherin-6B (Fig. 3B). It maybe is due to a low efficiency of shRNA-mediated knockdown. However, the Lim-1/2 expression domains (i.e. dI2, 4, 6) were unaffected by shRNA-mediated knockdown of Cadherin-6B (Fig. 4). Importantly, Cadherin-6B deficiency affected neither cellular proliferation (Fig. S2) nor apoptosis [20] in the neural tube. These results suggest that Cadherin-6B is specifically required for the generation of the Islet-1-expressing subgroup of interneurons, but not other classes of dorsal interneurons, in the chick neural tube. However, it is still uncertain whether Cadherin-6B controls the generation of dI3 via BMP signaling or other mechanisms.

4. Discussion

As described in this study, loss of Cadherin-6B in the dorsal neural tube resulted in the loss of the Islet-1-positive subgroup of the dorsal interneurons. However, the loss of the Islet1 population accompanied neither the compensatory expansion of other interneurons, such as the Lim-1/2 positive subgroups, nor their patterning in the neural tube. Thus, Cadherin-6B is required for the generation of Islet-expressing interneurons, but not for the patterning of dorsal neural progenitors in the embryonic chick.

This work builds on our previous findings that Cadherin-6B is able to regulate BMP signal in the neural tube. More importantly,

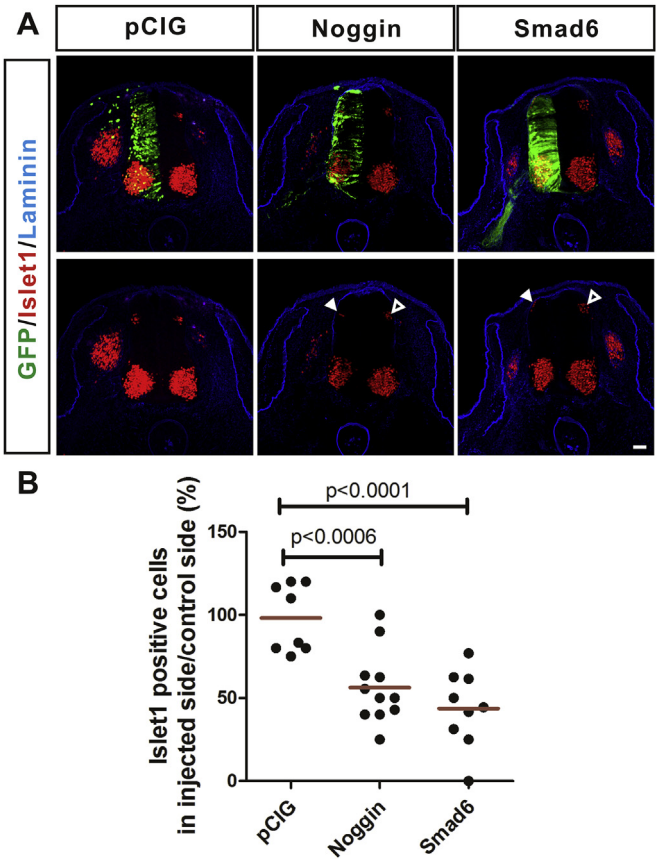

Fig. 2. Inhibition of BMP signaling caused a decrease in the size of the Islet-1-positive dorsal interneuron population. (A–B) Embryos (13-15 somite pair stage) were electroporated with overexpression constructs for Noggin or Smad-6. GFP expression from an IRES-GFP marks electroporated cells. Islet-1 and Laminin were stained in red and blue, respectively. Closed arrowheads and open arrowheads respectively indicate Islet-1-positive dorsal cells in the electroporated side and in the control side. pCIG (a control vector, n = 3 embryos), Noggin (n = 3 embryos), Smad6 (n = 3 embryos). Representative images are shown in A. The percentage represents the number of Islet-1 positive dorsal cells in the electroporated side divided by the number of Islet-1 positive dorsal cells in the control side (B, mean values are shown in red and p values were calculated with Student's t-test). Eight to 11 sections per each experimental group were analyzed. Scale bar = 25 μm.

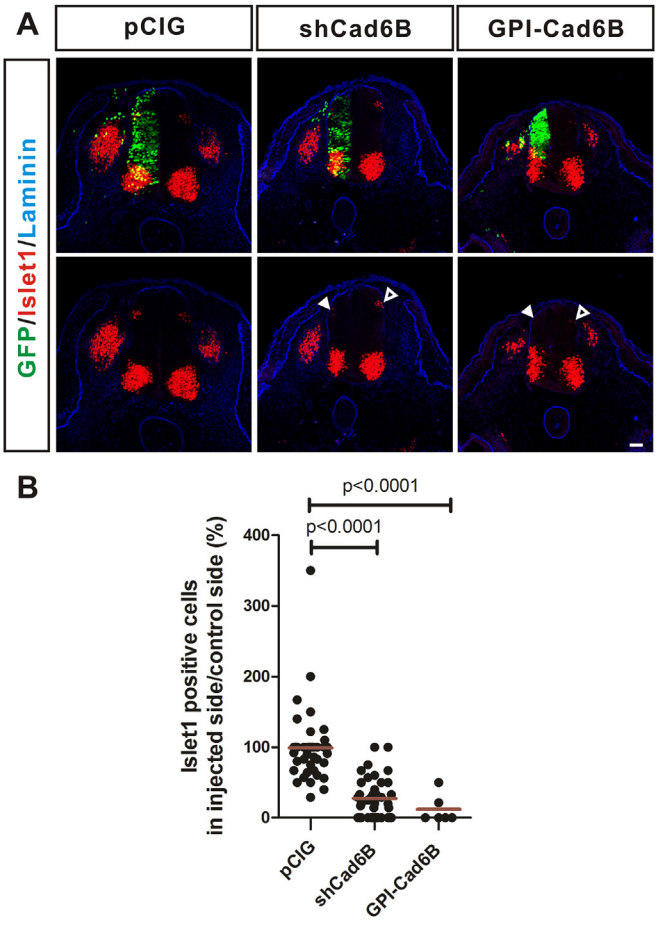

Fig. 3. Knockdown of Cadherin-6B led to a decrease in the generation of Islet-1-positive dorsal interneurons. (A–B) Embryos (13-15 somite pair stage) were electroporated with either a control vector (pCIG, n = 5 embryos), a mixture of pCIG and shRNA expressing vector targeting *cadherin-6B* (shCad6B, n = 5 embryos), or a construct expressing the dominant negative mutant form of Cadherin-6B (GPI-Cad6B, n = 4 embryos). GFP expression from an IRES-GFP marks electroporated cells. Immunostaining of Islet-1 and Laminin were performed in red and blue, respectively. Closed arrowheads and open arrowheads denote Islet-1 positive dorsal cells in the electroporated side and in the control side, respectively. Representative images are shown in A. The percentage represents the number of Islet-1 positive dorsal cells in the electroporated side divided by the number of Islet-1 positive dorsal cells in the control side (B, mean values are shown in red and p values were calculated with Student's t-test). Six to 43 sections per each experimental group were analyzed. Scale bar = 25 μm.

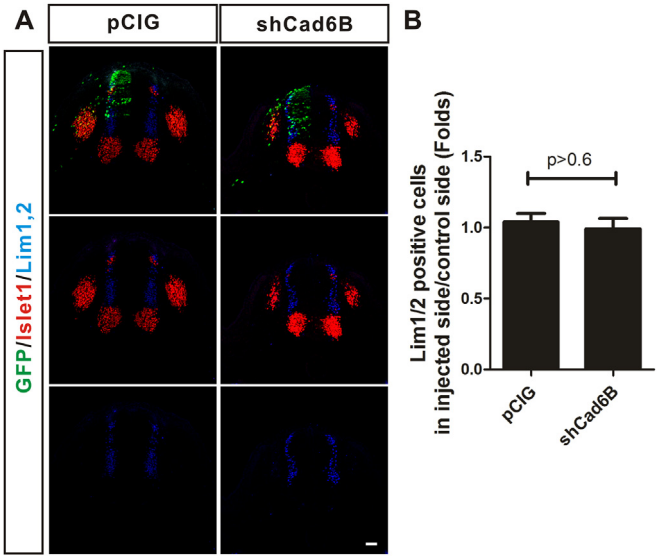

Fig. 4. Knockdown of Cadherin-6B did not affect patterning of the Lim-1/2 domain in the neural tube. (A–B) Embryos (13-15 somite pair stage) were electroporated with either a control vector (pCIG, n = 5 embryos) or a mixture of pCIG and shRNA-expressing vector targeting *cadherin-6B* (shCad6B, n = 5 embryos). Immunostaining of Islet-1 (red) and Lim-1/2 (blue) was performed. Representative images are shown in A. The folds represents the number of Lim-1/2 positive dorsal cells in the electroporated side divided by the number of Lim-1/2 positive dorsal cells in the control side (B, results are shown in mean ± SD and p value was calculated with Student's t-test). Scale bar = 25 μm.

we provide novel evidence that Cadherin-6B is required for Islet-1 interneuron generation. BMP is also known to be an essential regulator of interneuron specification in the neural tube. Specifically, ectodermal BMP induces the differentiation of the neural crest [11,24] and the generation of the dorsal middle cells of the roof plate [5]. Subsequently, various dorsal neural tube-derived ligands of the TGF-β superfamily, such as BMP-4, mediate the generation/patterning of dorsal interneurons [5] and also the emigration of the neural crest cells from the dorsal neural tube [12]. Importantly, the dorsal neural tube also expresses Cadherin-6B at this stage [12], wherein the local expression of Cadherin-6B is induced by ectodermal BMP expression [24]. Moreover, Cadherin-6B intrinsically activates BMP signaling independently of BMP ligands [17]. Indeed, Cadherin-6B is required for the overall emigration of the neural crest [20], as we verified in Fig. 1. In our current study, we have further shown that Cadherin-6B is additionally required for the generation of Islet-1-expressing dorsal interneurons.

The mechanisms by which Cadherin-6B regulates the generation of the dorsal interneurons are yet to be fully elucidated. The generation of the distinct interneuron subtypes in the dorsal neural tube is determined at multiple stages of their development. First, neural progenitors residing near the midline are patterned into cell type specific domains along the dorsal ventral axis of the neural tube and progenitors then migrate laterally, before fully differentiating into distinct interneuron subtypes [25]. It is therefore possible that Cadherin-6B plays a role in the lateral migration of interneuron progenitors. However, another possibility is that BMP signaling is spatially regulated in a population of cells expressing Cadherin-6B and that intrinsic regulation of BMP signaling in these cells might mediate the patterned expression of homeobox and helix-loop-helix transcription factors, which are key regulators of interneuron specification [23]. We will examine this issue in future work.

Recently, it was shown that FatJ cadherin is required for the patterned generation of Lim-1/2-expressing interneurons by regulating the size of the neural progenitor pool via YAP, a mediator of the Hippo pathway [26]. Loss of Cadherin-6B function mediated by ectopic expression of our dominant negative construct did not grossly affect cellular proliferation or apoptosis of neural progenitors in the neural tube compared to control (data now shown). However, it would be compelling to check whether Cadherin-6B also plays a role in the regulation of the cellular proliferation in each specific group of progenitors and each patterning domain in the neural tube.

Conflict of interest

The authors disclose no conflicts.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.136.

Transparency document

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