Molecular and Cellular Neuroscience 72 (2016) 114–122

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Runx1 contributes to the functional switching of bone morphogenetic protein 4 (BMP4) from neurite outgrowth promoting to suppressing in dorsal root ganglion

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ARTICLE INFO

Article history:
Received 12 August 2015
Revised 10 February 2016
Accepted 11 February 2016
Available online 15 February 2016

Keywords:
Runx
Transcription factor
Dorsal root ganglion
BMP4
Neurite outgrowth
CGRP

ABSTRACT

The runt-related transcription factor Runx1 regulates cell-type specification and axonal projections of nociceptive dorsal root ganglion (DRG) neurons, whereas bone morphogenetic protein 4 (BMP4) is required for axonal growth during neuronal development. Although Runx1 has been shown to be involved in BMP4 signaling in non-neural tissues, the Runx1 function in BMP4-dependent regulation of neuronal development is unclear. To investigate interactions between Runx1 and BMP4 in neurite outgrowth, we cultured DRGs from wild-type and *Runx1*-deficient mouse embryos in the presence or absence of BMP4. Neurite outgrowth was decreased in BMP4-treated wild-type DRGs and untreated *Runx1*-deficient DRGs, suggesting the inhibitory effect of BMP4 and facilitatory effect of Runx1 on neurite outgrowth. In addition, the combination of BMP4 treatment and Runx1 deficiency increased neurite outgrowth, suggesting that Runx1 is required for BMP4-induced suppression of neurite outgrowth and that the loss of Runx1 results in a functional switch of BMP4 from neurite growth suppressing to neurite growth promoting. Both BMP4 treatment and Runx1 deficiency increased calcitonin gene-related peptide (CGRP)-positive neurons, and CGRP expression was not increased by BMP4 treatment in *Runx1*-deficient mice, suggesting that Runx1 contributes to BMP4-induced CGRP expression in DRG neurons. Thus, Runx1 contributes to BMP4 regulation of neurite outgrowth and CGRP expression in DRG and may control BMP4 functional switching during embryogenesis.

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1. Introduction

Runt-related (Runx) genes encode the DNA-binding α-subunit of the runt domain transcription factor family. In mammals, three Runx family transcription factors play crucial roles in the development of neural and non-neural cells (for reviews, Ito, 2008; Stifani and Ma, 2009; Zagami et al., 2009). The functions of Runx1 in subtype specification from bipotential or multipotential progenitor cells have been demonstrated during the development of sensory neurons (for reviews, Inoue et al., 2008; Lallemend and Ernfors, 2012; Liu and Ma, 2011). Runx1 is expressed initially in TrkA-positive small DRG neurons at early developmental stages (Levanon et al., 2001; Marmigère et al., 2006) and is involved in the cell-type specification of TrkA-negative non-peptidergic and TrkA-positive peptidergic nociceptive neurons by promoting c-ret expression (Chen et al., 2006b; Yoshikawa et al., 2007) and by repressing TrkA and calcitonin gene-related peptide (CGRP) expression. In addition to neuronal subtype specification, Runx1 is involved in neurite outgrowth and axonal projection from sensory and motor neurons (Chen et al., 2006b; Marmigère et al., 2006; Yoshikawa et al., 2007, 2015; Yang et al., 2013).

Bone morphogenetic proteins (BMPs), including BMP4, are involved in neural development and regeneration. BMP signals are mediated by Smad-dependent and Smad-independent pathways (Miyazono et al., 2005, 2010). Receptor-regulated Smads (R-Smads: Smad1, Smad5, and Smad8) are involved in the Smad-dependent pathway, while LIMK1, which regulates actin dynamics, is involved in the Smad-independent pathways (Foletta et al., 2003; Lee-Hoeflich et al., 2004). It has been reported that BMPs regulate axonal growth *via* R-Smad and LIMK1 pathways (Benavente et al., 2012; Matsuura et al., 2007; Parikh et al., 2011; Phan et al., 2010; Zou et al., 2009). The actions of BMPs are bidirectional depending on the signaling pathways activated because R-Smads promote neurite outgrowth and regeneration (Finelli et al., 2013; Hazen et al., 2012; Parikh et al., 2011; Zou et al.,

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http://dx.doi.org/10.1016/j.mcn.2016.02.001
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2009), while LIMK1 inhibits neurite outgrowth (Benavente et al., 2012; Matsuura et al., 2007; Phan et al., 2010; Yamauchi et al., 2013). R-Smads and Smad4 interact with transcription factors to regulate target gene expression (Miyazono et al., 2005, 2010). This signaling cascade is modulated by inhibitory Smads (Smad6 and Smad7) (Miyazono et al., 2005, 2010).

Runx1 interacts with Smads and is involved in BMP signaling in hematopoietic cells (Knezevic et al., 2011; Miyazono et al., 2005, 2010; Pimanda et al., 2007). Runx1 is also a known downstream target of BMP4 (Knezevic et al., 2011; Pimanda et al., 2007); however, the functions of Runx1 in BMP4 signaling within the nervous system remain unclear.

In the present study, we examined the roles of Runx1 in BMP4-mediated neurite formation and CGRP expression in embryonic DRG neurons using *Runx1*-deficient mice *in vitro*.

2. Materials and methods

2.1. Animals

The function of Runx1 was examined in transgenic *Runx1*-deficient (*Runx1*$^{-/-}$::*Tg*) mice in which *GATA-1*-expressing hematopoietic cells are rescued by the *G1-HRD*-regulated expression of *Runx1*, whereas Runx1 expression remains deleted in other cells, such as sensory and motor neurons (Yokomizo et al., 2006; Yoshikawa et al., 2007). Although *Runx1*$^{-/-}$ mice die by E11.5 because of impaired fetal liver hematopoiesis (Okuda et al., 1996; Theriault et al., 2004, 2005; Wang et al., 1996), *Runx1*$^{-/-}$::*Tg* mice survive until the late embryonic stages, and thus we were able to analyze the roles of Runx1 in the development and axonal projections of DRGs. *Runx1*$^{+/+}$::*Tg* littermates were used as controls. Wild-type C57BL/6J mouse embryos (Japan SLC, Inc., Japan) were also used to examine the expression pattern of Runx1. All experiments were approved by the Animal Care Committee of the University of Tsukuba.

2.2. Explant culture of DRGs and quantification of neurite outgrowth

Explant cultures were prepared as described previously (Masuda et al., 2003, 2007) with some modifications. DRG explants from E13.5 embryos were cultured on BD Matrigel™ Matrix (BD Biosciences) in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with GlutaMAX™ (Invitrogen), 50 ng/ml nerve growth factor (NGF, Wako Pure Chemical Industries), 10% heat-inactivated fetal bovine serum (FBS, GIBCO), and 40 mM glucose. Bone morphogenetic protein 4 (BMP4, Wako Pure Chemical Industries) was added to the medium at 10 ng/ml. Neurites from the explants were detected by whole-mount immunostaining against β-tubulin. After 1 day in culture, the explants were fixed in 4% paraformaldehyde solution, rinsed with 0.1 M phosphate buffer (pH 7.4), and incubated overnight at room temperature (RT) with mouse anti-β-tubulin antibody (Promega; 1:200), followed by biotinylated anti-mouse IgG (Vector Laboratories; 1:200). Fixed immunolabeled explants were then incubated with peroxidase conjugated avidin–biotin complex (Vector Laboratories; 1:100) and the labeling visualized using the ImmunoPure Metal Enhanced DAB (3,3′-diaminobenzidine) Substrate Kit (Pierce). Slides were mounted in glycerol gelatin (Sigma-Aldrich) and Z-stack images of the stained neurites were captured using a confocal microscope (LSM510 META ver3.2, Carl Zeiss) with a 20× objective. The Z-stack images (1-μm step size) were then converted into a fully-focused image. Quantification of neurites was performed as described previously (Masuda et al., 2003, 2007). Neurite length and number were measured using ImageJ (NIH) and expressed relative to the values of untreated (no BMP4) DRG cultures from *Runx1*$^{+/+}$::*Tg* mice. All analyses were conducted by an investigator blinded to the treatment groups.

2.3. Dissociation culture of DRG neurons

DRGs from E13.5 embryos were dissociated by treatment with 0.05% trypsin–EDTA (Sigma-Aldrich) for 3 min at 37 °C and subsequent trituration. Next, 6–8 × 10^4 cells/ml were seeded in 16-well chambers on slides (Nunc) coated with poly-L-lysine (Sigma-Aldrich) and laminin (Sigma-Aldrich). Cultures were incubated at 37 °C in DMEM containing 10% heat-inactivated FBS. After 8 h, the medium was replaced with serum-free DMEM plus B-27 medium supplement (GIBCO), 3 mM L-glutamine (GIBCO), 250 U/ml penicillin G plus 25 ng/ml streptomycin (Celox Laboratories, Inc.), and 25 ng/ml NGF (basal medium). BMP4 was added to the medium at 1 ng/ml. Three days after plating, DRG neurons were fixed in 4% paraformaldehyde for 30 min at RT and immunostained with mouse anti-β-tubulin (Promega; 1:5000) or rabbit anti-calcitonin gene-related peptide (CGRP) (Chemicon; 1:6000). Slides were mounted in aqueous mounting medium (Permaflour, Beckman Coulter) and neurons examined under a confocal laser scanning microscope (LSM510 META ver3.2) with a 20× objective. Neuron number and neurite width were quantified using the Zeiss LSM image browser (Carl Zeiss) and expressed relative to the corresponding values of untreated dissociated DRG neurons from *Runx1*$^{+/+}$::*Tg* mice. All analyses were conducted by an investigator blinded to the treatment groups.

2.4. Immunohistochemistry

The following antibodies were used for immunohistochemical analysis: rabbit anti-Runx1 (Sigma-Aldrich; 1:1000), mouse anti-Runx1 (a gift from Dr. Ito, Cancer Science Institute of Singapore, National University of Singapore; 1:500), rabbit anti-CGRP (Chemicon; 1:4000), goat anti-CGRP (Biogenesis; 1:2000), and rabbit anti-calcitonin receptor-like receptor (CRLR) (Acris Antibodies; 1:100). Sections (12 μm thickness) were prepared on a cryostat (HM 500 OM, MICROM International GmbH), and if required, they were subjected to heat-induced epitope retrieval by heating to 105 °C for 5 min in Dako REAL™ Target Retrieval Solution (10×) (Dako) before immunostaining. Sections were incubated overnight at 4 °C with one of the primary antibodies in blocking solution, followed by incubation in a biotinylated secondary antibody for 1 h at RT. The sections were incubated with peroxidase conjugated avidin–biotin complex (1:100) for 30 min at RT and immunolabeling visualized using the ImmunoPure Metal Enhanced DAB Substrate Kit. For double staining, cryostat sections were incubated with mouse anti-Runx1 antibody and rabbit anti-CGRP, or goat anti-CGRP and rabbit anti-CRLR, followed by incubation with Alexa Fluor 488-labeled and 594-labeled secondary antibodies. Images were captured using a confocal microscope (Carl Zeiss). Both *Runx1*$^{-/-}$::*Tg* and *Runx1*$^{+/+}$::*Tg* tissues from the littermates were simultaneously processed.

2.5. Cell size analysis of DRG neurons

Serial transverse sections (12 μm) were cut using a cryostat and were stained with primary antibody against CGRP. The areas of individual CGRP-positive DRG neurons were measured in every 3rd section of whole E17.5 10th thoracic DRG using AxioVision imaging software (Carl Zeiss). Only cross-sectional areas of cell bodies containing a distinct cell nucleus were recorded.

2.6. Skin RNA isolation and cDNA synthesis

Total RNA was extracted from the back skin of E17.5 *Runx1*$^{-/-}$::*Tg* and *Runx1*$^{+/+}$::*Tg* littermates using TaKaRa RNAiso (Takara Bio Inc.). The total concentration of RNA was measured by spectrophotometry. cDNA was synthesized from 1 μg of total RNA using QuantiTect Reverse Transcription (QIAGEN) and then used as a template for gene cloning by PCR.

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