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BmpR1A is a major type 1 BMP receptor for BMP-Smad signaling during skull development

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ABSTRACT

Craniosynostosis is caused by premature fusion of one or more sutures in an infant skull, resulting in abnormal facial features. The molecular and cellular mechanisms by which genetic mutations cause craniosynostosis are incompletely characterized, and many of the causative genes for diverse types of syndromic craniosynostosis have not yet been identified. We previously demonstrated that augmentation of BMP signaling mediated by a constitutively active BMP type IA receptor (*ca*-BmpR1A) in neural crest cells (ca1A hereafter) causes craniosynostosis and superimposition of heterozygous null mutation of *Bmpr1a* rescues premature suture fusion (ca1A;1aH hereafter). In this study, we superimposed heterozygous null mutations of the other two BMP type I receptors, *Bmpr1b* and *Acvr1* (ca1A;1bH and ca1A;AcH respectively hereafter) to further dissect involvement of BMP-Smad signaling. Unlike caA1;1aH, ca1A;1bH and ca1A;AcH did not restore the craniosynostosis phenotypes. In our *in vivo* study, Smad-dependent BMP signaling was decreased to normal levels in mut;1aH mice. However, BMP receptor-regulated Smads (R-Smads; pSmad1/5/9 hereafter) levels were comparable between ca1A, ca1A;1bH and ca1A;AcH mice, and elevated compared to control mice. *Bmpr1a*, *Bmpr1b* and *Acvr1* null cells were used to examine potential mechanisms underlying the differences in ability of heterozygosity for *Bmpr1a vs. Bmpr1b* or *Acvr1* to rescue the mut phenotype. pSmad1/5/9 level was undetectable in *Bmpr1a* homozygous null cells while pSmad1/5/9 levels did not decrease in *Bmpr1b* or *Acvr1* homozygous null cells. Taken together, our study indicates that different levels of expression and subsequent activation of Smad signaling differentially contribute each BMP type I receptor to BMP-Smad signaling and craniofacial development. These results also suggest differential involvement of each type 1 receptor in pathogenesis of syndromic craniosynostoses.

1. Introduction

Craniofacial sutures are actively growing sites supporting the continuous growth of facial and calvarial bones. Premature fusion of sutures leads to craniosynostosis. Occurring in approximately 1 in 2500 live births, craniosynostosis leads to abnormal growth of calvarial bones, increases pressure inside the skull, which affects vision and in the extreme cases, may affect brain development (Mishina and Snider, 2014; Morriss-Kay and Wilkie, 2005; Snider and Mishina, 2014; Wilkie and Morriss-Kay, 2001). Studies in the past have successfully linked craniosynostosis to more than 20 genetic mutations; however, 70% of observed cases have no known genetic etiology (Lajeunie et al., 1995; Mishina and Snider, 2014; Twigg and Wilkie, 2015; Wilkie et al., 2010; Wilkie and Morriss-Kay, 2001). Therefore, it is necessary for further analysis to uncover genetic and pathophysiological mechanisms for diagnosis and development of treatments.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF- β) super family, play important roles in embryonic development, including craniofacial and calvarial bone development (Ishii et al., 2005; Kim et al., 1998; Liu et al., 2007; Urist, 1965). Activation of BMP signaling is through ligand-induced heterotertrameric complex formation. These complexes include type 1 and type II serine threonine kinase receptors. Heterotetrameric complex formation will thereafter lead to activation of type I receptor kinases, which phosphorylate R-Smads (de Caestecker, 2004; Heldin et al., 1997). Phosphorylated Smads will then accumulate into the nucleus where they function as transcriptional co-regulators. Genetics studies have linked BMP signaling with craniofacial and calvarial bone

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development. Particularly, our recent work demonstrated that a small increase of BMP-Smad signaling in mouse cranial neural crest leads to pre-mature fusion of the anterior frontal suture, which can be rescued by *Bmpr1a* heterozygosity (Komatsu et al., 2013).

BMP type I receptors play indispensable roles in transducing BMP signaling. In mammals, each BMP type I receptor has a distinct role during embryogenesis. For example, germ-line knockout of *Bmpr1a* or *Acvr1* leads to embryonic lethality at different stages of gastrulation (Gu et al., 1999; Mishina et al., 1999, 1995); germ-line knockout of *Bmpr1b* has no lethal impact during embryogenesis (Baur et al., 2000; Yi et al., 2000). On the other hand, during chondrogenesis, all three types of BMP type I receptors play overlapping roles; double and triple mutations of these receptors show more severe phenotypes in chondrogenesis than the loss of a single BMP type I receptor (Rigueur et al., 2015; Yoon et al., 2005, 2006). Despite the evidence that all BMP type I receptors are expressed during craniofacial and skull development, there is no knowledge available regarding how each of them is involved in transducing BMP signaling in these tissues during development. Particularly, it is unknown whether BmpR1B or AcvR1 are involved in regulating BMP signaling in craniosynostosis.

In this current study, we investigated the functions of BMP signaling mediated by each type I receptor during craniofacial development and potential involvement in pathogenesis of craniosynostosis by introducing heterozygous null mutations of each BMP type I receptor to the craniosynostosis model mouse we have reported (Hayano et al., 2015; Komatsu et al., 2013). The *in vivo* studies to rescue craniosynostosis phenotypes and subsequent *in vitro* studies to contribute BMP-Smad signaling activity demonstrated that BmpR1A plays distinct and major roles in skull development and in pathogenesis of craniosynostosis.

2. Materials and methods

2.1. Mouse breeding and isolation of primary osteoblasts

The mouse line carrying the Cre-inducible constitutively active *Bmpr1a* (*ca-Bmpr1a*, hereafter) transgene was described previously (Kamiya et al., 2008b; Komatsu et al., 2013). We first crossed $Bmpr1a^{+/-}$ (Mishina et al., 1995), $Bmpr1b^{+/-}$ (Yi et al., 2000) and $Acvr1^{+/-}$ (Mishina et al., 1999) mice with *P0-Cre* mice (*C57BL/6J-Tg(P0-Cre)94Imeg* (ID 148) provided by CARD, Kumamoto University, Japan) (Yamauchi et al., 1999) to generate mice $Bmpr1a^{+/-}$;*P0-Cre*, $Bmpr1b^{+/-}$;*P0-Cre* and $Acvr1^{+/-}$;*P0-Cre* mice. Subsequently, these mice were bred with *ca-Bmpr1a* mice to obtain *ca-Bmpr1a*;*P0-Cre*;$Bmpr1a^{+/-}$ (ca1A;1aH, hereafter), *ca-Bmpr1a*;*P0-Cre*;$Bmpr1b^{+/-}$ (ca1A;1bH, hereafter) and *ca-Bmpr1a*;*P0-Cre*;$Acvr1^{+/-}$ (ca1A;AcH, hereafter) mice. Littermates that did not carry either *ca-Bmpr1a* or *P0-Cre* and were wild type for the endogenous type I receptors were used as controls (Cont, hereafter). Littermates that carried *ca-Bmpr1a* and *P0-Cre* but were wild type for the endogenous type I receptors were used as mutants (ca1A, hereafter). We interbred $Bmpr1b^{+/-}$ mice to generate homozygous null mutant osteoblasts from newborn calvaria (Mansukhani et al., 2000; Yu et al., 2005). For *Bmpr1a* and *Acvr1*, we used the following conditional mutant mice to generate homozygous null osteoblasts: *Bmpr1a* floxed mice (Mishina et al., 2002), *Acvr1* floxed mice (Kaartinen et al., 2004), and Ubiquitin-Cre ^{ERT2} mice (No. 008085, The Jackson Laboratory) were intercrossed in order to isolate primary osteoblasts from newborn calvaria of which the genotype was either $Bmpr1a^{fx/-}$;*Ubi-Cre* ^{ERT2};*R26R*/+ or $Acvr1a^{fx/-}$;*Ubi-Cre* ^{ERT2};*R26R*/+. Cre activity was induced in culture by adding tamoxifen (100 ng/ml) for 6 days to convert the floxed allele to the Cre-recombined null allele (designated as dE). The degree of Cre-dependent recombination was monitored using the R26R Cre reporter allele (Soriano, 1999), and subsequently quantified by genomic Q-PCR, directly measuring the deleted exons. All mice were maintained on a mixed 129S6 and C57BL6/J background. They were housed in cages in a 20 °C room with a 12-h light/dark cycle. All animal experiments were performed in accordance with the policy and federal law of judicious use of vertebrate animals as approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

3. Micro-computed tomography (μCT)

Skulls were dissected, cleaned of extra tissue, and fixed with 10% formalin overnight. Whole heads were embedded in 1% agarose, placed in a 19 mm diameter tube, and scanned over their entire length using a micro-CT system (μCT40 Scanco Medical). Scan settings were: voxel size 10 μm, medium resolution, 70 kVp, 114 μA, 0.5 mm AL filter, and integration time 500 ms. Surface images were generated as described previously (Komatsu et al., 2013).

4. Histology, skeletal staining and picrosirious staining

Tissue fixation and section preparation was performed as described previously (Komatsu et al., 2013). The skulls dissected by postnatal day 9 were decalcified in 14% EDTA solution. Hematoxylin & Eosin and Von Kossa staining were performed according to standard protocols. Cranial bones were stained with alizarin red and alcian blue by a standard method (Hogan, 1994; Komatsu et al., 2013). Picrosirius red staining for sections was performed the standard procedure as described previously (Stern et al., 2012). Samples were observed under a polarized light microscope (Olympus BX51 microscope) and photographed.

5. Cell apoptosis and proliferation assays, immunohistochemistry

Apoptosis in calvaria at E18.5 stage was evaluated *via* Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay according to the instruction of ApopTag Red in Situ Apoptosis Detection Kit (Millipore, #S7165). In brief, frozen sections were subsequently heated in citrate buffer in the water bath for 5 min and then were immersed in 5% Donkey serum for 60 min. Slides were incubated with TUNEL reaction mixture at 37 °C, 60 min in the dark then incubated in Anti-digoxigenin conjugate rhododamine 30 min. After washing with PBS, slides were mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen, #P36931). Sections were examined under a fluorescence microscope (Olympus) with a TRITC set filter and positive cells were counted in the frontal suture and the frontal bone. Statistical analysis was performed using Student's T-test. Significance was accepted at $p < 0.05$.

Cell proliferation in E18.5 calvaria was detected *via* examining presence of Ki67. Slides were incubated with an anti-ki67 antibody (Cell Signaling, #D3D5, 1:500) for overnight at 4 °C, and incubated with an anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen, #A-21206, 1:200) for 1 h at room temperature. Sections were mounted with ProLong Gold antifade reagent with DAPI; Invitrogen. We counted the number of Ki67 positive cells in the frontal suture and the frontal bones. Statistical analysis was performed using Student's T-test. Significance was accepted at $p < 0.05$.

For immunohistochemistry, embryos were fixed in 4% PFA at 4 °C for 2 h, incubated in 30% sucrose/PBS at 4 °C overnight, embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan), and serially sectioned at 10 μm. The following antibodies were used in our study: pSmad1/5/9 (Cell Signaling, #13820, 1:100), and E11/Podoplanin (Santa cruz, #sc-53533, 1:100). The samples were incubated with these primary antibodies at 4 °C overnight. Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen, #A21206, 1:100) for pSmad1/5/9 and Alexa Fluor 488 goat anti-hamster IgG (Invitrogen, #A21110, 1:100) for E11/Podoplanin were used as secondary antibodies. Sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, #P36935).