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Altering Glypican-1 levels modulates canonical Wnt signaling during trigeminal placode development

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ABSTRACT

Glypicans are conserved cell surface heparan sulfate proteoglycans expressed in a spatiotemporally regulated manner in many developing tissues including the nervous system. Here, we show that Glypican-1 (GPC1) is expressed by trigeminal placode cells as they ingress and contribute to trigeminal sensory neurons in the chick embryo. Either expression of full-length or truncated GPC1 in vivo causes defects in trigeminal gangliogenesis in a manner that requires heparan sulfate side chains. This leads to either abnormal placodal differentiation or organization, respectively, with near complete loss of the ophthalmic (OpV) trigeminal ganglion in the most severe cases after overexpression of full-length GPC1. Interestingly, modulating GPC1 alters levels of endogenous Wnt signaling activity in the forming trigeminal ganglion, as indicated by Wnt reporter expression. Accordingly, GPC1 overexpression phenocopies Wnt inhibition in causing loss of OpV placodal neurons. Furthermore, increased Wnt activity rescues the effects of GPC1 overexpression. Taken together, these results suggest that appropriate levels of GPC1 are essential for proper regulation of canonical Wnt signaling during differentiation and organization of trigeminal placodal cells into ganglia.

Introduction

Glypicans (GPCs) are a conserved family of cell surface heparan sulfate proteoglycans (HSPGs) that modulate major signaling pathways during embryonic development of fruit flies to mammals (Fico et al., 2007; Filmus et al., 2008). Heparan sulfate side chains, attached to the core protein at conserved serine residues near the membrane anchored glycosyl-phosphatidylinositol (GPI) linkage, are thought to facilitate binding of growth factors and ligands, including Wingless/Wnt, Dpp/BMP, Fgf, and Hh, and are considered ligand carriers or coreceptors. Accordingly, functional perturbation of glypicans has been shown to cause significant defects in cell fate, cell movements, survival, and proliferation in mice, *Xenopus laevis*, *Drosophila*, and zebrafish (Filmus and Song, 2000; De Cat and David, 2001; Lin, 2004; Fico et al., 2007).

During vertebrate development, glypicans are expressed in a spatiotemporally regulated manner in the nervous system and other tissues (Niu et al., 1996; Saunders et al., 1997; Litwack et al., 1998; Luxardi et al., 2007). Their expression changes in pathological conditions, such as cancer. GPC3 and/or GPC1 are down-regulated in some ovarian cancer and mesothelioma cell lines (Lin et al., 1999; Murthy et al., 2000) while upregulated in others (e.g., pancreatic tumors) (Kleeff et al., 1998; Filmus, 2001; Matsuda et al., 2001; Su et al., 2006). Six glypican (GPC1–6) family members have been identified in mammals, two in *Drosophila melanogaster* (Dally and Dally-like), and two *in Caenorhabiditis elegans* (gpn-1 and lon-2) (Fico et al., 2007). Loss-of-function mutations in OCI-5/GPC3 in humans cause Simpson–Golabi–Behmel syndrome (SGBS), characterized by pre- and post-natal overgrowth, visceral and skeletal defects, and an increased risk of tumors (Pilia et al., 1996). A GPC3-deficient mouse model exhibits a similar phenotype (Cano-Gauci et al., 1999). Of the six glypicans in mammals, GPC1 is the most abundantly expressed in the developing brain, in both neuroepithelial precursors and differentiated neurons (Karthikeyan et al., 1994; Litwack et al., 1994, 1998) and functions in neurogenesis of the central nervous system (Jen et al., 2009). However, its potential role in patterning and formation of the peripheral nervous system has yet to be explored.

Here, we show that Glypican-1 is expressed by ectodermal and ingressing chick trigeminal placode cells at the time they differentiate into neurons and assemble into ganglia. Modulation of GPC1 levels by expression of full-length GPC1 or a truncated form that is thought to act in a dominant-negative fashion prevents placodal differentiation or proper ganglion formation, respectively, with particularly strong effects on the ophthalmic lobe. Consistent with studies showing that Wnt signaling is important for differentiation of ophthalmic trigeminal placodes (Lassiter et al., 2007; Canning et al., 2008), we find that GPC1 regulates Wnt activity in OpV ganglion formation. The results suggest that proper levels of GPC1 are critical for appropriate modulation of canonical Wnt signaling for differentiation and assembly of trigeminal placodes into ganglia.

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Materials and methods

Embryos

Fertilized chicken (*Gallus gallus domesticus*) eggs were obtained from local commercial sources and incubated at 37 °C to the desired stages.

In situ hybridization

cDNA plasmid obtained from BBSRC (ChickEST clone 418p2) was used to transcribe antisense riboprobe against chick Glypican-1. The plasmid was sequenced and found to contain the coding sequence of the chick Glypican-1 gene (NCBI accession number: XM_422590.1) corresponding to nucleotides 1233–2107. Whole-mount chick in situ hybridization was performed as described (Shiau et al., 2008). Embryos were imaged and subsequently sectioned at 12 μm.

Immunohistochemistry

Primary antibodies used were: anti-TuJ1 (Covance; 1:250), anti-HNK-1 (American Type Culture; 1:3–1:5), anti-GFP to recognize GFP signal after in situ hybridization (Molecular Probes; 1:150–1:250), anti-Islet1 (DSHB, clone 40.2D6; 1:150–1:250), and anti-active-caspase-3 (Promega; 1:150–250). Appropriate secondary antibodies against the subtype of the primary antibodies were conjugated to Alexa Fluor 488, 568, or 350 dyes (Molecular Probes). Images were taken on a Zeiss Axioskop2 plus fluorescence microscope, and processed using Adobe Photoshop CS3.

In ovo electroporation of the trigeminal ectoderm

Plasmid constructs were targeted to the presumptive trigeminal placodal ectoderm at the approximate axial level between the posterior forebrain and anterior hindbrain at 5 somites stage (stage 8+) up to stage 11. Immediately after injection, platinum electrodes were placed vertically across the chick embryo delivering current pulses of 5×8 V in 50 ms at 100 ms intervals as described (Shiau et al., 2008). Targeting DNA to the ectoderm resulted in transfection of the trigeminal placodes in the ectoderm and subsequently placode-derived cells that detach from the ectoderm and migrate into the ganglion anlage. The operated eggs were sealed and incubated at 37 °C for later analysis. Incubation times were ~16–24 hours to reach stages 13–14, ~ 24–36 hours to reach stages 15–16, ~ 40–48 hours to reach stages 17–18, and ~50–60 hours to reach stage 19.

Plasmid constructs

Full-length chick Glypican-1 cDNA (clone CS5) was isolated from a 4- to 12-somite stage chick macroarray library (Gammill and Bronner-Fraser, 2002). To create cytopcig-FL-GPC1, the full-length coding sequence (1.65 kb) was amplified from the library clone CS5 by PCR using forward and reverse primers corresponding to the coding sequence with flanking *Xho*I and *Cla*I site, respectively. Gene fragment was directionally inserted into the cytopcig vector (Shiau et al., 2008) at the *Xho*I/*Cla*I sites. Three mutant GPC1 constructs were made by a single-step or fusion PCR amplification off the full-length sequence, and modified gene fragment was directionally cloned into the *Xho*I and *Cla*I sites in the cytopcig vector. These are 1) cytopcig–GPC1–GPI which encodes the first 517 amino acids (1.55 kb) with a premature stop codon which eliminates the GPI-anchoring domain, 2) cytopcig–GPC1–ΔHS which has a 18-bp deletion that spans a coding region of six residues, SGSGSG (483–488 aa), containing three tandemly positioned putative glycosaminoglycan (GAG) attachment sites (serine residues 483, 485, 487) based on sequence annotation in UniProtKB for chick GPC1 protein (accession P50593), and 3) cytopcig–GPC1–ΔGPI–ΔHS which has deletions of both the GPI-anchor domain and the putative HS sites. Two versions of full-length and mutant constructs were made: one without myc–tag fusion and one with a 6× myc–tag inserted at *Cla*I/*Eco*RV sites in frame with the coding sequence at the C-terminus, which was used to validate protein expression of the constructs. Both versions were tested and determined to give the same effects on ganglion development, albeit FL-GPC1 was more potent without the myc–tag. Thus, most experiments with the full-length and mutant expressions were conducted with the construct lacking the myc–tag. Dominant-active β-catenin construct was made in the pCIG vector with IRES nuclear localized GFP as previously described (Megason and McMahon, 2002; Lassiter et al., 2007), and RFP-Wnt reporter (also named pTOP-nDSRed2) (gift from Dr. Andy Groves (Lassiter et al., 2007)) was a modified version of the TOPGAL construct (DasGupta and Fuchs, 1999) where the reporter gene was replaced with RFP.

BrdU treatment

Electroporated embryos were screened and selected for broad GFP expression in the trigeminal region at stage 14 for both control and experimental cases. Each embryo was explanted into an individual well and treated with 0.1 mM BrdU in Ringer's solution at 37 °C for 30 minutes. Embryos were then fixed in 4% paraformaldehyde overnight at 4 °C, washed in PBS, incubated in 2N HCl in PBS for 30 minutes, followed by 0.1 M perboric acid (H_3BO_4) for 10 minutes, washed in PBS several times, and processed for cryosectioning and immunostaining with the mouse anti-BrdU (Sigma, B2531; 1:150–1:250).

Quantification of the area of trigeminal ganglion

An outline of the ganglion as marked by TuJ1 staining was made by the freehand selection tool on whole mount grayscale fluorescent images in the ImageJ software. All images were taken with the same setup using a 5× objective on the Zeiss Axioskop2 plus microscope and at the same image size, with the entire ganglion in focus. The area of the ganglion outline was determined by the area measurement function in ImageJ with the scale calibrated to the actual length.

Results

Expression of Glypican-1 mRNA in the trigeminal placodes and other embryonic tissues during early chick development

As a first step in examining the possible developmental role of Glypican-1 (GPC1), we first characterized its mRNA expression in the chick embryo at stages 9–18 by whole mount in situ hybridization (Fig. 1 and data not shown). This corresponds to the time window of early trigeminal development starting from neural crest migration to ganglion condensation. No expression of GPC1 mRNA was noted in the trigeminal neural crest cells, derived from the midbrain and anterior hindbrain (rhombomeres 1 and 2) levels of the neural tube, from stage 9 through gangliogenesis (Figs. 1A–C, F, L). Interestingly, we find that GPC1 is expressed by the presumptive trigeminal placodal ectoderm starting at approximately stage 12, coincident with the beginning of placodal differentiation and ingression but not earlier (Figs. 1C and D). GPC1 expression persists later as placodal cells assemble and condense into ganglion at stages 14–16 (Figs. 1F and L) and later at stage 18 (data not shown). GPC1 is expressed by both the ophthalmic (OpV) and maxillomandibular (MmV) placodes that form the trigeminal ganglion (Figs. 1F and L). To confirm that these GPC1 expressing cells are in fact placode-derived, we labeled the placodal ectoderm with GFP by in ovo electroporation prior to placodal ingression. Embryos were then collected at later stages after placodal cells have begun to delaminate from the ectoderm. GPC1 expression was detected by in situ hybridization in the GFP-expressing placodal