

Appendix A

Cell surface heparan sulfate proteoglycan Glypican-1 and placodal ganglion formation

A.1 Abstract

Biochemical studies have implicated a specific interaction between a cell surface heparan sulfate proteoglycan (HSPG), Glypican-1 (GPC1), and Slits in rat brain lysate (Liang et al., 1999; Ronca et al., 2001). However, the link between glypicans and Slit-Robo signaling in vivo during development remains elusive. More specifically, the mechanism of the Slit1 distribution and its reception by Robo2 on trigeminal placodal neurons may rely on a co-receptor, such as GPC1, but this process is unknown. To more deeply investigate the nature of Slit1–Robo2 signaling, we sought to characterize the potential role of GPC1 during cranial sensory ganglia formation. In this chapter, we show that GPC1 mRNA is expressed in the placodal cells at the right time and place for a role during cranial gangliogenesis, and additionally the expression of GPC1 on early migrating hindbrain neural crest cells also indicate a possibility for its role in the neural crest. GPC1 gain-of-function caused both reduced and disorganized placodal neurons at early assembly and later severely reduced trigeminal ganglion at times of condensation. In extreme cases, this caused elimination of nearly the entire ophthalmic lobe of the ganglion. The data so far suggest that proper regulation of GPC1 expression is essential for placodal ganglion formation and that GPC1 function is sufficient to affect placodal cell survival and/or proliferation, and possibly morphogenesis. In summary, the results show a previously unknown expression and potential role for a glypican family member in both neural crest and placodal development. These provide an exciting outlook for further exploration on the

function of GPC1 and the general role of HSPGs in neural crest and cranial sensory ganglia development.

A.2 Introduction

Glypicans (GPCs) constitute one of two major families of cell surface heparan sulfate proteoglycans (HSPGs). The other family is the syndecans which are transmembrane proteins as compared to the glypicans which are anchored to the membrane through a glycosyl-phosphatidylinositol (GPI) link. There are six members of glypicans (GPC1-6) in mammals, two in *Drosophila melanogaster* (*Dally* and *Dally-like*), two in *Caenorhabditis elegans* (*gpn-1* and *lon-2*), and one in zebrafish (*knypek*) (Fico et al., 2007) which have so far been identified. The core protein of the glypican molecules are well conserved which consist of a large globular cysteine-rich domain, a smaller domain encompassing the heparan sulfate (HS) attachment sites, and a sequence signal for the GPI attachment (Filmus and Song, 2000). On the glypican core protein, HS side chains are attached near the membrane anchor and are thought to facilitate binding of heparin-binding growth factors and ligands. Therefore, glypicans are considered co-receptors for various signals and possibly act to regulate the distribution and activity of these factors. Additionally, however, there is also a possibility for glypican functions independent of its HS side chain. Overexpression of GPC3 can induce apoptosis in a cell line specific manner but this does not require its HS chains (Gonzalez et al., 1998). Furthermore, multiple types of post-translational processing can take place on glypicans, such as complex modifications

of the HS chains and proteolytic cleavage at the GPI anchor or at the N-terminal cysteine-rich domain, which can yield tissue-specific as well as non-cell-autonomous effects as reviewed in (Fico et al., 2007).

During vertebrate development, glypicans are known to be expressed in a spatiotemporally regulated manner in the nervous system as well as in various other tissues (Litwack et al., 1998; Luxardi et al., 2007; Niu et al., 1996; Saunders et al., 1997). Their expression also changes in pathological cases, such as cancer. GPC3 and/or GPC1 have been found to be either downregulated in some ovarian cancer and mesothelioma cell lines (Lin et al., 1999; Murthy et al., 2000) or upregulated in others (e.g. pancreatic tumors) (Filmus, 2001; Kleeff et al., 1998; Matsuda et al., 2001; Su et al., 2006).

The functions of proteoglycans during embryonic development are profound. Functional perturbation of glypicans in mice, *Xenopus laevis*, *Drosophila*, and zebrafish has been shown to affect Wingless/Wnt, Dpp/BMP, Fgf and/or Hh signaling in affecting cell fates, body size, cell movements (e.g. during gastrulation), cell survival and proliferation (Fico et al., 2007; Filmus and Song, 2000). Loss of function mutations in OCI-5/GPC3 in humans cause the Simpson-Golabi-Behmel syndrome (SGBS), which is characterized by pre- and post- natal overgrowth and visceral and skeletal defects and an increased risk for tumors (Pilia et al, 1996). *GPC3* knockout mouse model also exhibit similar phenotypes (Cano-Gauci et al., 1999). The first *Drosophila* homolog for glypican, *division abnormally delayed* (*dally*) gene, was found in a screen for defects in cell division patterning in the forming CNS (Nakato et al., 1995). *dally* mutants have delayed G2-M transition in dividing cells in the eye disc and lamina as well as defects in morphogenesis of adult tissues (i.e. the eye, antenna, wing, and genitalia) and viability. *Dally*-like, the second glypican member in *Drosophila*, has been shown to facilitate long range Wingless signaling by transporting the signal to neighboring cells in the wing imaginal disc, while *Dally* acts as classical co-receptor (Franch-Marro et al., 2005). In terms of regulation of cell

proliferation, the effects of glypican in vertebrates appear to be the opposite of that in the fly, such that glypican-3 is a negative regulator whereas dally promotes cell division. Taken together, genetic evidence from glypican mutants implicate functions for glypican in regulating cell survival, proliferation, and/or morphogenesis, likely reflecting its association with a wide range of major signaling pathways. How specificity is conferred in a spatiotemporal manner that link the function of a proteoglycan to a particular signaling pathway on a cell remains to be uncovered.

Recent evidence have implicated direct interaction of heparan sulfate proteoglycans (HSPGs) with Slit for its function (reviewed in (Hohenester, 2008)). Structural studies have suggested a Slit–Robo–HS complex based on the result that the second leucine rich repeat of Slit binds to heparin and both can bind to Robo (Fukuhara et al., 2008; Hussain et al., 2006). Disruption of heparan sulfate (HS) chains by heparinase decreases affinity of Slit–Robo binding and also blocks Slit repulsive activity in vitro (Hu, 2001; Piper et al., 2006). Consistent with this, when excess HS is applied to compete with endogenous HS, Slit activity is also compromised in a growth cone collapse assay (Piper et al., 2006). Genetic evidence from *Drosophila*, also indicate the interaction of syndecan, a HSPG, with Slit–Robo in regulating distribution and efficiency of Slit signaling (Johnson et al., 2004; Steigemann et al., 2004). In vertebrates, the role of syndecan with Slit–Robo has not been characterized, but recombinant vertebrate Glypican-1 has been found to bind specifically to Slit1 and Slit2 from rat brain extracts in a heparan sulfate dependent manner (Liang et al., 1999; Ronca et al., 2001). The functional relationship of HSPG and Slit–Robo in vivo is unknown.

We have previously identified the critical role of Slit1–Robo2 in mediating neural crest-placode assembly of the trigeminal ganglion as presented in Chapter 2. The nature of the slit ligand reception and distribution required for proper gangliogenesis remains unexplored. The biochemical interaction reported for Glypican-1 and Slit1 prompted us to

wonder if there may be a possible connection between GPC1 and Slit1–Robo2 signaling during trigeminal gangliogenesis. The expression and function of glypicans during cranial gangliogenesis have not been investigated previously. As a first step, we sought to explore whether Glypican-1 may be involved in neural crest and/or placode development. We characterized the gene expression pattern of Glypican-1 during cranial gangliogenesis and we found that trigeminal and epibranchial placodal cells and early hindbrain neural crest cells express Glypican-1 at the time of ganglion assembly and crest migration, respectively. The results show a previously unknown expression of a glypican family member which is potentially involved in both neural crest and placodal development. Furthermore the proper expression level of Glypican-1 in the placodal tissue appears to be crucial for formation of the trigeminal ganglion. Overexpression of GPC1 causes both reduced and disorganized placodal neurons at early assembly and severely reduced trigeminal ganglion later at times of coalescence. In extreme cases, this caused elimination of nearly the entire ophthalmic lobe of the ganglion. The data so far suggest that proper regulation of GPC1 expression is essential for placodal ganglion formation and provide a basis for further exploration on its function in neural crest and cranial sensory ganglia development.

A.3 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 according to the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1992).

In situ hybridization

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

Immunohistochemistry

Whole chick embryos were fixed in 4% paraformaldehyde overnight at 4°C, washed in PBT (PBS + 0.2% tween) and either immunostained as whole embryos and/or processed for 10 μm cryostat sections. Primary antibodies used were anti-HNK-1 (American Type Culture; 1:3 or 1:5), anti-Islet1 (DSHB, clone 40.2D6; 1:250), and anti-TuJ1 (Covance; 1:250). Secondary antibodies were obtained from Molecular Probes and used at 1:1000 or 1:2000 dilutions, except 1:250 dilution for Alexa Flour 350 conjugated antibodies. Images were taken using the AxioVision software from a Zeiss Axioskop2 plus fluorescence microscope, and processed using Adobe Photoshop CS3.

In ovo electroporation of the trigeminal ectoderm

DNA was injected overlying the presumptive trigeminal placodal ectoderm at stages 8–10 by air pressure using a glass micropipette. Platinum electrodes were placed vertically across the chick embryo delivering $5 \times 8 \text{ V}$ in 50 ms at 100 ms intervals current pulses. Electroporated eggs were re-sealed and re-incubated at 37°C to reach the desired stages (i.e. 30–36 hours to stages 15–16 and 36–48 hours to stages 17–19).

Plasmid constructs

Full length chick Glypican-1 cDNA (clone CS5) was isolated from a 4– to 12– somites stage chick macroarray library as previously described (Gammill and Bronner-Fraser, 2002). The coding sequence (1.65 kb) was amplified from the library clone by PCR using a 5' primer with a flanking a XhoI site and a 3' primer with a flanking ClaI site. This fragment was inserted into PCRII-Topo vector using the TOPO TA cloning kit (Invitrogen) and clone G1 (PCRII-Topo + GPC1, size = 5.65 kb) was determined to be correct by sequencing. The fragment was then digested at the XhoI/ClaI sites and directionally cloned into the XhoI/ClaI sites in the cyto-pcig vector, yielding clones CG2 and CG3 (cytopcig + GPC1, size = 7.8 kb) which were determined to be correct by sequencing.

A.4 Results and discussions

A.4.1 Expression of Glypican-1 mRNA in the precursors of cranial sensory ganglia suggests its potential role in ganglion formation

To investigate whether Glypican-1 (GPC1) may have a role during cranial gangliogenesis, I have characterized the mRNA expression of GPC1 in the chick embryo at stages 9–18 by whole mount in situ hybridization (Fig. 1). This represents the time window prior to or at the beginning of neural crest migration up to ganglion condensation. In the presumptive trigeminal region, the neural crest cells migrating at the midbrain and anterior hindbrain (R1/R2) level starting at stage 9 through stages 13–14 do not appear to express

GPC1 (Fig. 1, A-C, E). At stages 12–13, low levels of GPC1 were sometimes detected in the ectoderm bilateral to the midbrain region in a salt-and-pepper pattern indicative that it may be in trigeminal placodes (data not shown) but this remains to be confirmed. Later, at stages 14–16 during trigeminal ganglion assembly, GPC1 is expressed by both the ophthalmic (OpV) and maxillo-mandibular (MmV) placodes that form the trigeminal ganglion (Fig. 1, E-F). To confirm that these GPC1 expressing cells are in fact placode-derived, I have labeled the placodal ectoderm with GFP by ectoderm electroporation prior to its ingress and collected these embryos at later stages to process for GPC1 in situ hybridization. Results show that all GFP expressing placode-derived cells and discrete regions of the placodal ectoderm express GPC1 (Fig. 1, G-K), while they interact with the trigeminal neural crest cells which do not. The matching expression pattern of GPC1 and Robo2 as previously described (Shiau et al., 2008) in the trigeminal placodal cells lends a possibility for GPC1 to act as a co-receptor in regulating Slit1–Robo2. Alternatively, GPC1 may have an independent function during trigeminal gangliogenesis. This expression pattern may be conserved with the mammalian GPC1 as it is also expressed in the peripheral cranial and trunk sensory ganglia (i.e. trigeminal and dorsal root) in mouse and rat embryos (Litwack et al., 1998), though the distinction between the neural crest and placode cell types was not made.

The expression pattern is somewhat different in the hindbrain region corresponding to the presumptive epibranchial ganglia region, which is at around the second to the fourth branchial arches. Unlike the trigeminal neural crest, GPC1 is detected in the hindbrain neural crest cells during migration (Fig. 1, C-D), albeit expression is transient, since by stage 14, it is downregulated (Fig. 1, L-M). In addition, GPC1 is expressed in the epibranchial placodal ectoderm at later stages 14–16 (Fig. 1, E-F and L-M). The expression pattern of GPC1 in the hindbrain region suggests that it may potentially have an early role in hindbrain neural crest migration and later in epibranchial placodal gangliogenesis.

A.4.2 Expression of Glypican-1 mRNA in other tissues, including the neural tube, otic vesicle, limb, and somite

Expression of GPC1 mRNA was found in several other tissues besides the neural crest and placodal cells at stages 9–18. GPC1 was weakly expressed in the forming neural tube and notochord throughout these stages at the cranial levels (Fig. 1, A-L). By stage 12, the otic placode is found to express GPC1 albeit weakly but later through stage 18, its expression is strong in the invaginating and forming otic vesicle (Fig. 1, C, E-F, L, P). The forming limb bud also expresses GPC1 (Fig. 1P). Among these, the tissue expressing GPC1 in the most dynamic manner is probably the somitic tissue. Through the stages examined (st. 9–18), the GPC1 mRNA appears to be expressed in a gradient fashion in the presomitic mesoderm (PSM) highest at the newly forming somites and decreases both rostrally (towards the more anterior somites) and caudally towards the tail of the embryo. The expression found for GPC1 may suggest potentially interesting functions both early in specifying segmentation of the anterior PSM and later in epithelialization or boundary formation of the newly forming somite. The different expression of GPC1 from that of Robo2 and Slit1 in the trunk region, such that Robo2 is expressed in the neural folds and restricted portions of the somites similar to Slit1 (data not shown) (De Bellard et al., 2003), suggests that GPC1 may interact with signaling pathways other than Slit–Robo in the somites (Fig. 1, N-P). These expression patterns of GPC1 were consistent with that found previously in the forming neural tube, somite, and limb (Niu et al., 1996). It is also important to note that since Niu et al. examined mostly different stages (st.7–12 and 20–25), we have identified several previously unknown expression patterns of GPC1, including its expression in the cranial ganglia. They also characterized expressions of GPC1 that were not studied here at later stages 20–25, including expression of GPC1 in the

mantle zone of the telencephalon, apical epidermal ridge, the proximal limb, atrioventricular canal, and in the heart outflow tract which they suggest is endothelial- and not neural crest- derived mesenchyme.

A.4.3 Overexpression of Glypican-1 causes early disorganization of trigeminal placodal neurons and later severely reduced ganglion

The expression of Glypican-1 in the placodal ectoderm and derived cells as the trigeminal ganglion assembles suggests a possibility for its function in placodal gangliogenesis. To begin to test the function of GPC1, I have designed a full-length chick Glypican-1 expression construct (cytopcig + GPC1) and introduced it in the trigeminal placodal ectoderm prior to ingressation at stages 8–10 to study the effects of GPC1 gain-of-function on ganglion formation. Strikingly, overexpression of GPC1 in the trigeminal placodal cells caused severe reduction in placodal cell number, giving rise to diminished ganglia, as well as effects on placodal assembly at early stages. At times of early ganglion assembly (stages 15–16), a significant number of transfected ganglia showed aberrant defects (77%, n=7/9). These defects were classified into two general groups: either both disorganized (aberrant positioning) and reduced in cell number (33%, n= 3/9) (Fig. 2D-F) or only appeared reduced in cell number (44%, n=4/9) (Fig. 2G-I). Later after the ganglion is well condensed at stages 17–19, the transfected ganglia with GPC1 overexpression were severely reduced in cell number and/or disorganized (69%, n=11/16). Out of the total number of ganglia exhibiting a phenotype, 82% (n=9/11) of the cases were diminished ganglia (Fig. 2M-O) of which 44% (n=4/9) had nearly complete loss of the ophthalmic (OpV) lobe (Fig. 2P-R). The loss of OpV phenotype thus constituted a significant 25% (n=4/16) of the total GPC1 transfected ganglia analyzed at stages 17–19. Control untransfected sides of the GPC1 electroporated embryos and control GFP electroporated

embryos did not show apparent ganglion abnormalities at all stages analyzed (Fig. 2, A-C and J-L and data not shown).

The results suggest an intriguing possibility that GPC1 may have a role in regulating placodal cell survival and/or proliferation and possibly also cell organization. The potential effect on cell proliferation may reflect a role for GPC1 on neuronal differentiation of the placodal cells, which takes place in the placodal ectoderm prior to ingress. This is based on the fact that most, if not all, placodal cells express neuronal markers by the time they ingress. Furthermore, it is intriguing to examine whether ingress may be affected such that a blocked migration may cause a reduced number of placodal neurons; however, this may be a secondary effect to loss of neuronal differentiation. Alternatively if undifferentiated placodal cells are aberrantly found to migrate to the ganglion anlage by GPC1 overexpression, this would demonstrate that migration and differentiation can be uncoupled, but this is unknown. Taken together, the data suggest that GPC1 function is sufficient to affect several aspects of the development of the placodal neurons during trigeminal ganglion formation, which likely involves various signaling pathways.

A.5 Conclusion and future work

The gene expression pattern and gain-of-function of GPC1 suggest its potential role in development of the trigeminal placodal ganglion. GPC1 was also expressed in the early migrating hindbrain neural crest cells and the epibranchial placodes but its role in these cells has not yet been examined. There are several lines of future research that would be

important to fully uncover the role of GPC1 in neural crest and placode development in the future.

First, understanding the cellular and molecular mechanisms mediated by GPC1 gain-of-function in causing the severe phenotype of trigeminal ganglion loss would provide important insights into the function of GPC1. It would be necessary to clarify the cellular effects as to whether this effect is on placodal cell survival, proliferation, and/or differentiation and also whether it alters placodal cell assembly. To identify the molecular mechanism responsible for mediating this function, it would be important to first identify the protein domain of GPC1 required for this phenotype, whether this requires the HS chain or not. Function of the core glypican protein has been suggested previously (i.e. transient expression of GPC-3 can induce apoptosis and this requires membrane anchorage but not the heparan sulfate chains (Gonzalez et al., 1998)). This suggests a potential role for GPC1 independent of its HS based co-receptor function. Overexpression of GPC1 may have an effect on Slit1 signaling or on other pathways (e.g. Wnt (Lassiter et al., 2007) and Fgf signaling (Stark et al., 2000)) implicated in trigeminal placodal development. Thus, the examination of whether this alters distribution of the protein expression patterns of these ligands and whether this may activate pathway specific downstream mediators would be revealing on the potential association of GPC1 to the different signaling pathways.

To further reveal its function, it would also be important to identify the subcellular localization and the potential proteolytic processing of the GPC1 proteins in these cells. Since an antibody against chick GPC1 that works for immunohistochemistry may not be available, the characterization of the exogenous GPC1 tagged with a reporter may yield useful information as to its localization on the cell membrane and/or possibly in the nucleus (which has been suggested but remains to be verified (Filmus and Song, 2000; Liang et al., 1997)) as well as its potential post-translational modifications (i.e. proteolytic cleavage at the GPI anchor or at a different site).

Gene knockdown of GPC1 in the trigeminal and epibranchial placodal cells as well as in the hindbrain neural crest would be necessary to uncover its endogenous role during development of the peripheral nervous system. Finally, the interaction between GPC1 and Slit1–Robo2 signaling can be tested by exploring whether altering GPC1 expression changes Slit1 expression and also if they are functionally interdependent (i.e. does gain or loss of function of Slit1 or Robo2 rescue the GPC1 mutant phenotype and vice versa). Further work on any of these future directions would undoubtedly provide new insights into the role of HSPG and its potential link with signaling pathways involved in placode and neural crest development.

Figure 1.

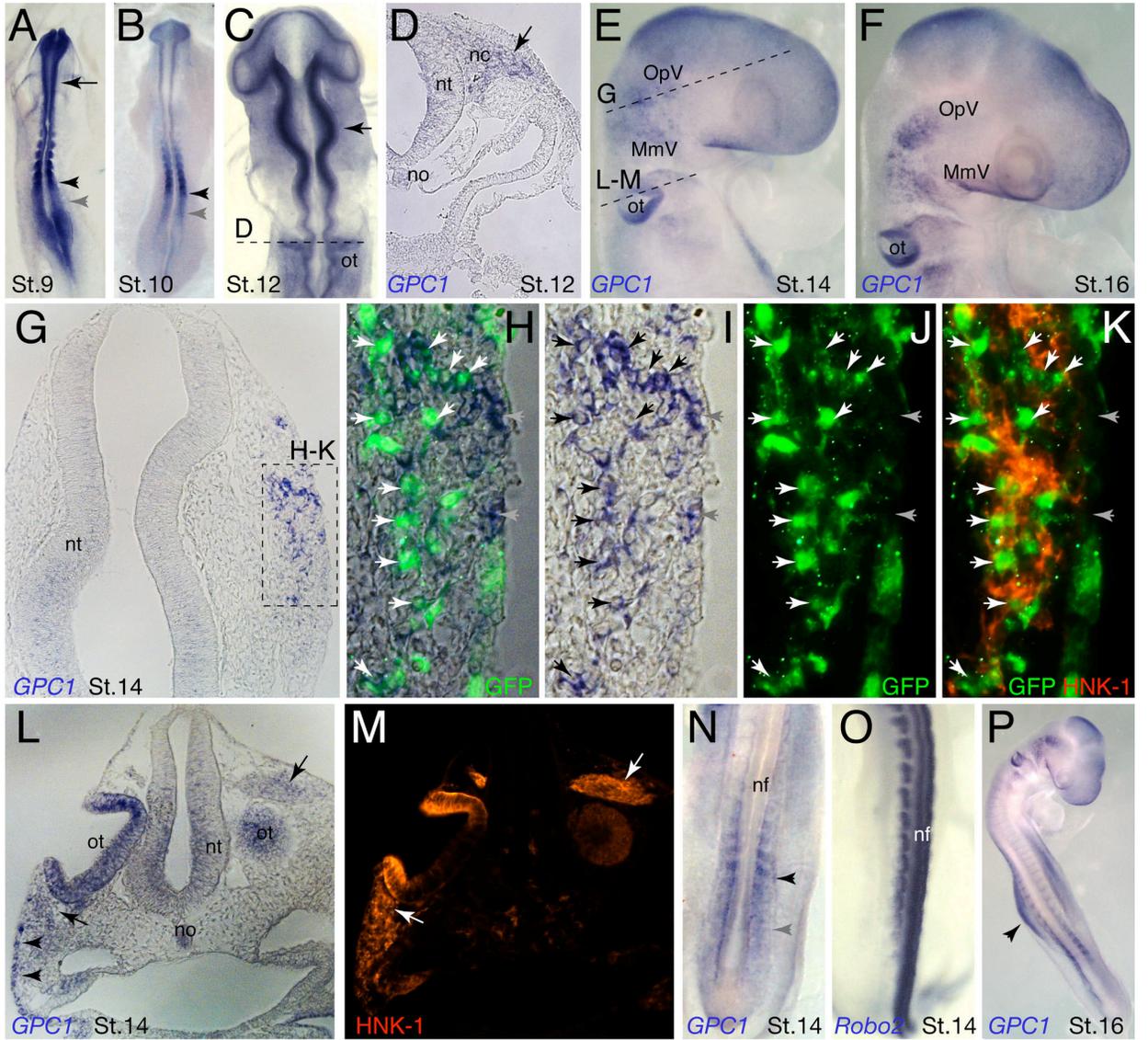


Figure 1. Glypican-1 mRNA expression in trigeminal and epibranchial placodes, hindbrain neural crest, and various other tissues. GPC1 mRNA expression is revealed by whole mount in situ hybridization using an antisense chick GPC1 riboprobe. **(A)** Stage 9 chick embryo revealing a dynamic rostrocaudal gradient of GPC1 expression in the presomitic mesoderm (PSM) (black and gray arrowheads) and a strong expression in the neural tube (arrow). **(B)** Stage 10 embryo revealing the same gradient expression in the PSM (black and gray arrowheads). **(C)** Stage 12 embryo showing expression in the neural tube (arrow) and in the hindbrain neural crest at rhombomere 4 (dotted line). **(D)** Cross section at the level indicated by the dotted line in C. **(E)** Stage 14 and **(F)** stage 16 embryos showing expression in the OpV and MmV trigeminal and epibranchial placodes and otic vesicle. **(G)** Cross section at the level indicated in E showing the expression of GPC1 in the OpV placodes (dotted box) and weakly in the neural tube. **(H)** Overlay image showing overlap of GFP expression in the ectoderm and ectoderm-derived cells and GPC1 in situ expression (white arrowheads), and expression of GPC1 in an area of the placodal ectoderm not electroporated by GFP vector (gray arrowheads). Images of the same section as in H showing **(I)** GPC1 expression alone, **(J)** GFP expression alone and **(K)** overlay of GFP and neural crest marker HNK-1 expression. **(L)** Cross section at the level indicated in E showing GPC1 expression in the epibranchial placode (arrowheads), weakly in the hindbrain neural crest (arrows), otic vesicle, neural tube, and notochord. **(M)** Same section as in L showing HNK-1 expression on the hindbrain neural crest cells that also express GPC1 (arrows). **(N)** Expression of GPC1 and **(O)** Robo2 mRNA in the trunk regions of stage 14 embryos showing a mostly non-overlapping pattern. **(P)** Whole mount stage 16 embryo showing GPC1 expression in the limb bud as well as all other tissues aforementioned. OpV, ophthalmic; MmV, maxillo-mandibular; ot, otic vesicle; nt, neural tube; no, notochord; nc, neural crest; nf, neural folds.

Figure 2.

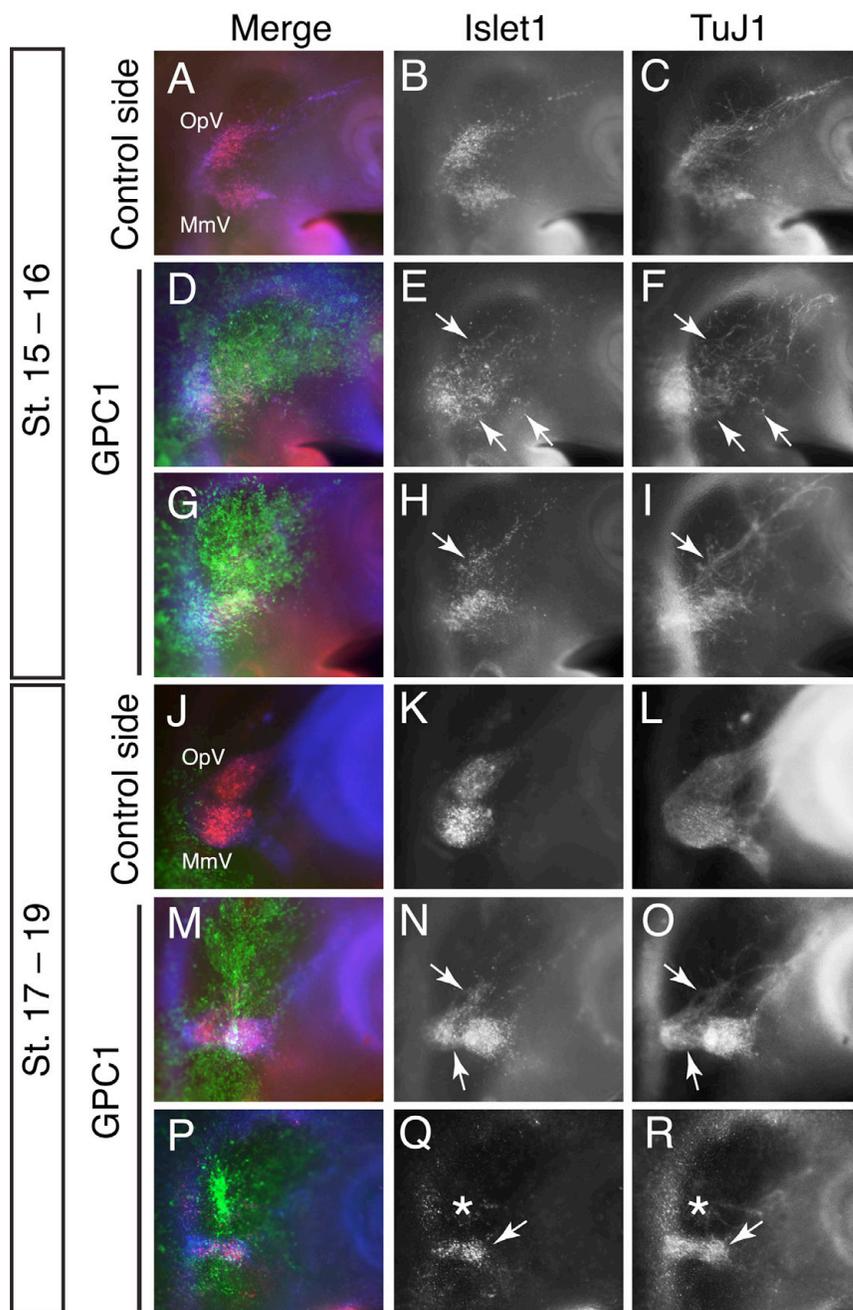


Figure 2. GPC1 overexpression in the trigeminal placodal ectoderm causes early disorganization of the placodal neurons and later severely reduced ganglion. Color overlay panel showing GFP (green) expression in area of transfection and double immunostaining for broad neuronal markers Islet1 (red) and TuJ1 (blue) to reveal placodal neuronal cell bodies and processes, respectively. Representative images of GPC1 electroporated embryos analyzed at stages 15-16 showing (A-C) a typical trigeminal ganglion on the control untransfected side, and aberrant GPC1 transfected ganglia which displayed two general categories of phenotypes: (D-F) severely disorganized and reduced population of placodal neurons (arrows) or (G-I) only markedly reduced population of the placodal neurons (arrow showing the OpV region). Later at stages 17-19, (J-L) control untransfected side (which had a small region of GFP expression but predominantly not in the ganglion region) showing normal ganglion formation, whereas the GPC1 transfected ganglia showed (M-O) dramatically reduced ganglion with some disorganization (arrows) and in extreme cases (P-R) near complete loss of the OpV lobe (asterisk) and a reduced MmV (arrow) ganglion. OpV, ophthalmic; MmV, maxillo-mandibular.