PRDC regulates placode neurogenesis in chick by modulating BMP signalling

Nadja N. Kriebitz, Clemens Kiecker, Laura McCormick, Andrew Lumsden, Anthony Graham, Esther Bell *

MRC Centre for Developmental Neurobiology, 4th Floor New Hunts House, Kings College London, Guy's Campus, London SE1 1UL, UK

A R T I C L E   I N F O

Article history:
Received for publication 29 April 2009
Revised 8 October 2009
Accepted 8 October 2009
Available online 15 October 2009

Keywords:
PRDC
BMP
Pharyngeal arches
Epibranclhal placodes
Endoderm
Neurogenesis

A B S T R A C T

The epibranchial placodes generate the neurons of the geniculate, petrosal, and nodose cranial sensory ganglia. Previously, it has been shown that bone morphogenetic proteins (BMPs) are involved in the formation of these structures. However, it has been unclear as to whether BMP signalling has an ongoing function in directing the later development of the epibranchial placodes, and how this signalling is regulated. Here, we demonstrate that BMPs maintain placodal neurogenesis and that their activity is modulated by a member of the Cerberus/Dan family of BMP antagonists, Protein Related to Dan and Cerberus (PRDC). We find that Bmp4 is expressed in the epibranchial placodes while Bmp7 and PRDC are expressed in the pharyngeal pouches. The timing and regional expression of these three genes suggest that Bmp7 is involved in inducing placode neurogenesis and Bmp4 in maintaining it and that BMP activity is modulated by PRDC. To investigate this hypothesis, we have performed both gain- and loss- of-function experiments with PRDC and find that it can modulate the BMP signals that induce epibranchial neurogenesis: a gain of PRDC function results in a loss of Bmp4 and hence placode neurogenesis is inhibited; conversely, a loss of PRDC function induces ectopic Bmp4 and an expansion of placode neurogenesis. This modulation is therefore necessary for the number and positioning of the epibranchial neurons.

© 2009 Elsevier Inc. All rights reserved.

Introduction

BMPs are the largest subgroup of the evolutionarily conserved transforming growth-factor-beta (TGFβ) superfamily (Wozney, 2002; Chen et al., 2004). Throughout embryonic development, their activity is regulated at several levels, both intracellularly and extracellularly (Munoz-Sanjuan and Brivanlou, 2002). Extracellularly, BMP activity is restricted by diffusible inhibitors that bind to BMP ligands and prevent them from activating their receptors (Munoz-Sanjuan and Brivanlou, 2002). The Cerberus/Dan superfamily is one group of extracellular BMP inhibitors, which includes Cerberus (Bouwmeester et al., 1996; Zhu et al., 1999), Dan (Dionne et al., 2001; Gerlach-Bank et al., 2002), Gremlin (Hsu et al., 1998; Bardot et al., 2001; Khokha et al., 2003), Coco (Bell et al., 2003), Caronte (Yokouchi et al., 1999), and PRDC (Minabe-Saegusa et al., 1998; Pearce et al., 1999).

BMPs have been shown to be involved in numerous developmental processes, including epibranchial placode neurogenesis (Hogan, 1996; Begbie et al., 1999; Ducy and Karsenty, 2000; Sudo et al., 2004; Holzschuh et al., 2005). The epibranchial ganglia, the geniculate, petrosal, and nodose, innervate the pharyngeal cavity and relay gustatory information from there to the solitary tract. Developmentally, these neurons originate from focal thickenings of the ectoderm, the epibranchial placodes (Webb and Noden, 1993). These structures develop as part of the pharyngeal metameres (Graham, 2007). They are induced to form by the pharyngeal pouches, and they come to lie just dorsal and posterior to the pouches. Much research has been done into how these placodes are initially established and a number of studies have highlighted the role of BMPs, Wnts, and FGFs in their formation (Begbie et al, 1999; Holzschuh et al, 2005; Nechiporuk et al, 2005, 2007; Nikaido et al, 2007; Sun et al, 2007; Freter et al, 2008). Fate map studies have demonstrated that both the epibranchial and otic placodes share a common domain very early in development (Streit, 2002), which is thought to be induced by FGF signalling (Nechiporuk et al, 2005, 2007). The subsequent delineation of otic and epibranchial fate is controlled by Wnt signalling: Wnt is required for the induction of the otic placode, whereas repression of Wnt signalling is required for epibranchial placode formation (Ohyama et al, 2006; Freter et al, 2008, reviewed by McCabe and Bronner-Fraser, 2009). Subsequently, BMPs have an important role in the induction of neurogenesis in the epibranchial placodes (Begbie et al, 1999; Holzschuh et al., 2005).

In chick, Bmp7 is expressed in the pharyngeal pouch endoderm and can induce epibranchial neurogenesis when added to isolated cranial ectoderm in vitro or by implantation of BMP7-soaked beads in vivo (Begbie et al, 1999; our unpublished observations). Conversely, the addition of follistatin, a BMP7 antagonist, can inhibit the ability of the pharyngeal endoderm to induce placode neurogenesis (Begbie et al, 1999). Similarly in fish, Bmp2b and Bmp5 are expressed in the endoderm of the arches and their expression is required to induce epibranchial neurogenesis (Holzschuh et al, 2005).

However, a number of important questions regarding the role of BMP signalling in epibranchial placode development remain open. First, Bmp7 is widely expressed in the pharyngeal endoderm, suggesting that spatial specificity in BMP signalling is mediated by...
other factors. To date, no known BMP inhibitors have been identified that have the correct temporal and spatial expression profile to fulfill this role (Bardot et al., 2001; Ogita et al., 2001; Gerlach-Bank et al., 2002; Müller et al., 2006; this work). Secondly, there has been little analysis of a possible role for ongoing BMP signalling in the development of the placodes. In particular, are BMPs required for the maintenance of epibranchial neurogenesis in addition to induction? An ongoing involvement of BMP signalling is seen in other developing systems: for example, BMPs have been shown to be involved in the induction, delamination, and differentiation of the neural crest (Liemi et al., 1995; Reissmann et al., 1996; Liu and Jessell, 1998).

To address these issues, we have analysed the role of BMP signalling at stages beyond the initial induction of the placodes. We investigated the expression of both Bmp4 and Bmp7 and a variety of their inhibitors to see if BMPs play a role in later epibranchial placode development and whether there are antagonists expressed at the correct time and place to restrict the spatial and/or temporal extent of BMP signalling. We show that, while the pharyngeal pouches continue to express Bmp7, the placodes themselves express Bmp4. Furthermore, we have identified a BMP antagonist, PRDC, that is restricted to the pharyngeal endoderm and whose expression domain abuts the placodal expression of Bmp4. PRDC is expressed in the correct temporal and spatial manner to restrict the expression of Bmp4, which in turn we propose is required for correct epibranchial placode specification. To test this, we overexpressed PRDC, which resulted in loss of Bmp4 and Bmp7 and loss of epibranchial neurogenesis. Conversely, loss of PRDC function increases Bmp4 expression (but not that of Bmp7) and results in increased epibranchial neurogenesis. Finally, we ectopically expressed BMP4 and found that the increase in placode neurogenesis is indeed due to the activity of BMP4. We thus demonstrate that PRDC provides a negative restraint on the activity of BMP4, resulting in a localised expression domain of Bmp4, which in turn is necessary for the spatiotemporal specificity of placode formation and maintenance.

Materials and methods

Cloning of chick PRDC and electroporation constructs

PRDC was cloned from stage 3 chick cDNA using primers from the 5′UTR: GCAGCCGGGACCCGGCCGAG and 3′ covering the stop codon: GAACAACTCAGGCAAGATGTGA (primers designed from sequence accession number XM_419552). The fragment was then isolated and subcloned into pBluescript, CS2+, and ires-GFP (internal ribosome entry site – GFP; Andreae et al., 2009) vectors.

To inhibit endogenous PRDC, siRNA (small interfering RNA) constructs were designed using the Ambion siRNA target finder. The following oligos were used at a final concentration of 1 μg/ml: (top) 5′-GATCCCC TTCAGAAGCTTGAGACGTT TTCAAGAGA CACCTGCTT CACCTTGAAATT TTATGAAAA-3′, (bottom) 5′-AGTTTTCACAAA AAATTCGAAGGTGAGAGTCG TTCTTGAA CAATCTGTCACCTTCTGAA CG-3′. The control siRNA had the same sequence but was scrambled: (top) 5′-GATCCG CTATGAGAGTGGAAGTCGA TTCAAGAGA CACCTGCTT CACCTTGAAATT TTATGAAAA-3′, (bottom) 5′-AGTTTTCACAAA AAATTCGAAGGTGAGAGTCG TTCTTGAA CAATCTGTCACCTTCTGAA CG-3′. The primer sets were cloned into ires-GFP.

Bmp4/GFP was constructed by cloning the full length Xenopus laevis Bmp4 (Wilson et al., 1997) into the ires-GFP vector using the primers 5′ gATCGATGACATCATGATGCTTGAAAC 3′ and 3′ TGGGGCCCTTTGACCTCAGATATCGATg 5′.

Chick in ovo electroporations

Embryos were staged according to Hamburger and Hamilton (1955). Eggs were windowed and electroporated at stage 9–10 (to target ectoderm) or stage 11 (to target endoderm) with 1.5 μg/μl of the appropriate expression construct (Kiecker and Lumsden, 2004). In order to target endoderm, DNA was injected directly into the tissue that will become the endoderm of the pharyngeal arches.

X. laevis injections and RT-PCR

Chick PRDC RNA was generated by linearising the CS2/PRDC construct with Ascl and transcribing with the mMessage mMachine SP6 kit from Ambion. Embryos were staged according to Nieuwkoop and Faber (1994) and injected for phenotype in the ventral marginal zone with 1 ng of RNA. For the animal cap experiments, embryos were injected in the animal pole at the 1 to 2 cell stage with combinations of 1 ng of PRDC, 1 ng of Bmp4/7 (Suzuki et al., 1997), or 200 pg of Wnt8 (Hoppler et al., 1996). RT-PCRs were performed at gastrula stage 11 using standard procedures (30 cycles; Wilson and Melton, 1994). Ornithine decarboxylase (ODC) was used as a loading control in these experiments.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisations were carried out on both chick embryos (Kiecker and Lumsden, 2004) and Xenopus embryos (Harland, 1991). Digoxigenin-labelled antisense RNA probe was synthesised from linearised plasmid pBS/PRDC (XbaI/T7). A NeuroM EST (Chisto268d13) was purchased from Geneservice. All other plasmids have been described previously (chick clones: Bmp4, Graham et al., 1994; Bmp7, Begbie et al., 1999; Pax2, Baker and Bronner-Fraser, 2000; Gremlin, Bardot et al., 2001; Sox3, Ishii et al., 2001; Delta1, Phox2a, Begbie et al., 2002; Dan, Gerlach-Bank et al., 2002; Xenopus clones: Ems1, Patarnello et al., 1997; Hoxd9, Wright et al., 1990; Krox20, Sham et al., 1993; Odb2, Panneese et al., 1995). Some embryos were embedded in 20% gelatin, fixed overnight at 4 °C in 4% PFA/PBS, and sectioned at 50 μm using a vibratome.

Immunohistochemistry

Whole-mount immunohistochemistry was carried out as previously described (Begbie et al., 1999) using the HuC (1:40; Invitrogen) and eGFP antibody (1:200; Kiecker and Lumsden, 2004).

Results

Expression of Bmp4, Bmp7, and their inhibitors in pharyngeal arch development

Bmp7 is expressed in the endoderm of the pharyngeal arches from stage 13 and acts to induce epibranchial neurogenesis (Fig. 1A; Begbie et al., 1999). We have analysed the expression of Bmp4 and find that, unlike Bmp7, it is expressed in the geniculate, petrosal, and nodose placodes from stage 15 (Fig. 1B and section in Fig. 1L). We also see a small patch of Bmp4 expression in the endoderm of the first pharyngeal arch. The endodermal expression of Bmp7 (red) stops immediately adjacent to the ectodermal Bmp4 expression (blue; see arrows in Fig. 1C). To confirm that Bmp4 is expressed within the placodes themselves, we analysed the expression of Pax2 (red) and Bmp4 (blue) in the same embryo (Fig. 1D). Pax2 is expressed within the epibranchial placodes (Baker and Bronner-Fraser, 2000) and we find that Bmp4 is expressed within this Pax2 domain (the blue Bmp4 is masking some of the red placodal expression of Pax2). Based on these temporal and spatial expression patterns, we propose that BMP7 is required to induce neurogenesis of the placodes and BMP4 is required to localize and maintain this process. This raises the question: how is the activity of BMP4 regulated to maintain its discrete expression domain?

We analysed the expression of BMP4 antagonists to see if we could find a local antagonist that might modulate the activity of the epibranchial expression of Bmp4. Both Dan (Gerlach-Bank et al., 2002; Fig. 1E) and Gremlin (Bardot et al., 2001; Fig. 1F), members of the Cerberus/Dan family, are expressed in the pharyngeal arches but their expression is...
restricted to the mesenchyme so although they are diffusible, they are unlikely to play a role in epibranchial placode specification.

We extended our analysis to other members of this gene family and isolated chik PRDC. We find that its expression is restricted to the endoderm of the pharyngeal arches. We detected PRDC expression by RT–PCR from Hamburger–Hamilton (HH) stage 3 (data not shown); however, we do not see expression by whole-mount in situ hybridisation in the embryo until stage 15. PRDC is predominantly expressed in the pharyngeal arches (Figs. 1G–I). Initially, weak PRDC expression can be seen in the first arch at stage 15 (data not shown). By stage 16, expression is stronger in the first arch (arrow, Fig. 1G) and weak in the second arch (arrowhead, Fig 1G). This expression quickly strengthens and expression is seen in the next posterior arch at stage 18/19 (data not shown). By stage 21, expression is detected strongly in the first, second, and third pharyngeal arches (Fig. 1H). However, this is transient and disappears by stage 25 (data not shown). Horizontal sections through a stage 21 embryo (Fig. 1I) illustrate that PRDC expression is restricted to the endoderm of the pouches, beginning in the most rostral pouch and then progressing more caudally. The expression stops where the endoderm abuts the ectoderm (arrow, Fig. 1I). In order to compare the endodermal expression of PRDC with its ligands, Bmp7 and Bmp4, we performed a set of double in situ hybridisations. We find that the expression of PRDC (red; Fig. 1J) overlaps with the expression of Bmp7 (blue). The expression of PRDC reaches further into the first arch than that of Bmp7 (arrow, Fig. 1J). Double in situ hybridisations of PRDC/Bmp4 show that the expression of PRDC (red) abuts the expression of Bmp4 (in blue; Fig. 1K). A horizontal section through an embryo that has been stained for both PRDC and Bmp4 (both in blue, Fig. 1L) demonstrates that the endodermal expression of PRDC is immediately adjacent to the ectodermal expression of Bmp4 (arrow).

PRDC can inhibit Bmp but not Wnt signalling in X. laevis

All members of the Cerberus/Dan gene family have been shown to inhibit BMPs and a small subsection of this family can also inhibit Wnts and Nodals (Bouwmeester et al., 1996; Bell et al., 2003; Avian-Krechmer and Hsieh, 2004). Previous research has demonstrated that PRDC can inhibit BMP2 and BMP4 in vitro (Sudo et al., 2004) but not whether it can also inhibit Wnt signalling. In order to investigate the specificity of PRDC and if it could also inhibit Bmp7 (in addition to Bmp2 and Bmp4), we injected chick PRDC RNA into the ventral marginal zone of 4-cell stage Xenopus embryos and left them to develop until late neurula stages. Embryos developed a partial secondary axis indicative of BMP inhibition (compare normal embryo in left panel to injected embryo in right panel, Fig. 2Aa; Niehrs, 2005). To assess the anterior/posterior (AP) level of these axes, we performed whole-mount in situ hybridisation with a variety of AP markers. We find expression of Hoxb9 (spinal cord; Fig. 2Aii; Wright et al., 1990), Krox20 (hindbrain; Fig. 2Aiii; Sham et al., 1993), and occasionally Otx2 (midbrain/forebrain; Fig. 2Aiv; Pannese et al., 1995), but not Ems1 (forebrain; white arrows indicate lack of Ems1 expression in the neural tissue of the secondary axis, Fig. 2Av; Patarnello et al., 1997). We do, however, see Ems1 expression in the presumptive kidneys of some of the secondary axis {see black arrow, 44% (n = 16) embryos}. This suggests that PRDC is capable of inhibiting BMP but the lack of forebrain structures indicates that Wnt signalling is not inhibited (Clinka et al., 1998; Piccolo et al., 1999).

To investigate this further, we coinjected PRDC with either Bmp4 or Bmp7, or Wnt8 in animal caps, and then analysed the induction of immediate downstream target genes of BMP and Wnt signalling by RT–PCR. Xbra and epidermal keratin are the immediate response genes of BMP4 and BMP7 (Bell et al., 2003). Coinjection of Bmp4 and Bmp7 with PRDC resulted in the inhibition of this induction (see arrows in top two gels, Fig. 2B), demonstrating that PRDC could inhibit both Bmp4 and Bmp7 in the animal cap assay. In contrast, we found that PRDC could not inhibit the induction of Siamois and Xnr3 by Wnt8 (bottom gel, Fig. 2B; Bell et al., 2003). These results confirm that PRDC can inhibit both Bmp4 and Bmp7 in vivo but not Wnts.

Therefore, we have identified a BMP inhibitor whose expression is restricted to the endoderm of the pharyngeal arches. The timing and location of Bmp4 and PRDC expression (both are expressed from stage 15) suggest an interaction and a specific role for these two genes in epibranchial placode specification. Bmp7 is expressed earlier, at a time when PRDC is not expressed and therefore must have a PRDC-independent function. These data suggest a pivotal role for PRDC, an antagonist of BMP signalling, in restricting the expression of Bmp4 (which is autoregulated; Balemans and Van Hul, 2002) in the epibranchial placodes to ensure correct placode formation and maintenance.

Loss of BMP activity inhibits placode formation in chick

To investigate this further, we overexpressed PRDC by electroporation in vivo and analysed the formation of the placodes. As BMPs are autoregulated and even though PRDC is an extracellular BMP inhibitor (Sudo et al., 2004), we would expect to detect a loss of Bmp expression at the RNA level after electroporation of PRDC. PRDC/GFP was electroporated into the epibranchial ectoderm at stage 9 and embryos were harvested up to 48 hours later between stages 17 and 21. We monitored the endogenous expression of both Bmp4 and Bmp7 to see whether their levels were modulated by exogenous PRDC (Figs. 3A–L). Control electroporations of GFP rarely showed changes in Bmp4 (89% normal (n = 9); Figs. 3A–C) or Bmp7 expression (82% normal (n = 11); Figs. 3G–I). When PRDC was electroporated, a loss of Bmp4 (83% (n = 24); Figs. 3D–F) and Bmp7 (56% (n = 18); Figs. 3J–L) expression correlated with the pattern of GFP expression (arrows in Figs. 3E, F, K, and L). A clear reduction in Bmp4 can be seen when comparing the electroporated side (E′) with the control side (D′). A loss of Bmp7 also correlated with the GFP (see arrowheads in K).

Next, we investigated the effect on the epibranchial placodes following PRDC overexpression by analysing a variety of placodal markers (Fig. 4). We looked first at the expression of Sox3, one of the earliest expressed placode markers. It is initially widely expressed but becomes restricted to the placodes themselves by stage 16 (Figs. 4A–F; Ishii et al., 2001). Ectopic PRDC caused a loss of expression of Sox3 that correlated with the expression of GFP (arrows Figs. 4E and F; 63% (n = 8)). Control electroporations of the ires–GFP construct alone rarely showed changes in Sox3 expression (82% normal (n = 11); Figs. 4A–C, arrows in B and C demonstrate that there is no loss of Sox3

Fig. 1. Expression of Bmp7, Bmp4, and their antagonists in chick pharyngeal arch development. (A) Expression of Bmp7 in the endoderm of the pharyngeal arches at stage 21. Arrows indicate where the pharyngeal endoderm contacts the ectoderm. (B) Bmp4 is expressed in the epibranchial placodes at stage 21. (C) Endodermal Bmp7 (in red) is expressed adjacent to the ectodermal expression of Bmp4 (in blue). Arrows demonstrate where the expression patterns abut. (D) Bmp4 (in blue) is expressed within the placodal domain of Pax2 (in red). White arrows show the Pax2 expression and black arrows the expression of both Pax2 and Bmp4. (E) A section through a stage 21 embryo shows Dan expression in the mesoderm of the first and second pharyngeal arches. (F) Gremlin has a similar expression pattern as shown in a section through a stage 23 embryo. (G) PRDC is expressed in the first pharyngeal arch at HH stage 16 (see arrow) and weakly in the second arch (see arrowhead). (H) Expression of PRDC at stage 21 is detected strongly in the first three arches and weak level expression can be seen in the fourth. Arrow highlights expression of PRDC under the eye. (I) Horizontal sections through a stage 21 embryo show that expression of PRDC is restricted to the endoderm (see arrow marking the endoderm/ectoderm border). (J) Bmp7 (in blue) is expressed within the PRDC domain. Arrow demonstrates where the expression of Bmp7 stops. (K) Two-colour whole-mount in situ shows PRDC expression in the endoderm (red) and Bmp4 in the placode (blue). Horizontal section through a double-DIG in situ reveals the expression of PRDC adjacent to the Bmp4 domain (L). Arrow illustrates where the endodermal expression of PRDC stops. 1st, first pharyngeal arch; 2nd, second pharyngeal arch; 3rd, third pharyngeal arch; ect, ectoderm; endo, endoderm; g, geniculate; mes, mesenchyme; n, nodose; p, petrosal; ov, otic vesicle.
where there is GFP). We next analysed the expression of Pax2, which is initially expressed in the pre-placodal region and then becomes restricted to the epibranchial placodes (Hidalgo-Sánchez et al., 2000; Figs. 4G–L). We never saw any change in Pax2 expression following overexpression of PRDC (compare L and K with the control side J; 100% normal (n = 9)). Control electroporations were also performed, and these were mainly normal (75% (n = 8); compare Figs. 4G and H). The fact that Pax2 expression did not change following expression of the BMP inhibitor PRDC suggests that initial specification and patterning of the placodes is normal and not affected by the suppression of endogenous Bmp.

We next analysed the expression of Delta1 (Figs. 4M–R), one of the earliest neurogenic markers of the placodes whose expression is restricted to cells within the placodal ectoderm (Begbie et al., 2002). Overexpression of PRDC resulted in a loss of Delta1 that correlated with the expression of PRDC/GFP. In this embryo, Delta1 expression was lost in both the geniculate and the petrosal placodes, demonstrating that the early neuronal precursors are not committed

Fig. 2. Overexpression of cPRDC in X. laevis. (A) Embryos were injected at the 4-cell stage in the ventral marginal zone with 1 ng chick PRDC RNA and left to develop until early tadpole stages. All control embryos are in the left hand panel, and PRDC-injected in the right, asterisk indicates secondary axis. (i) Uninjected control embryo compared to PRDC-injected embryo which has a partial secondary axis (marked with an *). Embryos were analysed for a variety of anterior–posterior markers: Hoxb9 (ii), Krox20 (iii), Otx2 (iv), and Emx1 (v). Arrows in (ii) and (iii) show the Hoxb9 and Krox20 gene expression in the secondary axis. White arrows in (v) indicate the lack of Emx1 forebrain expression, whereas the black arrow shows the kidney expression of Emx1. (B) PRDC can inhibit Bmp4 and Bmp7 but not Wnt8, as demonstrated by the co-injection of PRDC with these genes and the analysis of their immediate downstream targets. PRDC blocked the induction of brachyury (Xbra) and epidermal keratin (EK) by Bmp4 and 7, but not the induction of Siamois (Sia) and Xenopus nodal related-3 (Xnr3) by Wnt8 (see arrows). cg, cement gland; ey, eye; fb, forebrain; hb, hindbrain; ki, kidney; mb, midbrain; nc, neural crest; r3, rhombomere 3; r5, rhombomere 5; sc, spinal cord.
Fig. 3. Overexpression of PRDC leads to a loss of Bmp expression. Left panels (A, D, G, J) are control side of electroporated embryos; middle panels (B, E, H, K), electroporated side; and right panels (C, F, I, L), GFP of the electroporated construct. Electroporations were targeted to the ectoderm, all embryos are stage 20/21. (A–C) Effect of ires-GFP on Bmp4 expression. Arrow in B shows normal Bmp4 expression in the geniculate (compare A and B) and arrow in C shows that the ires-GFP construct was expressed in the same region. (D–F) Effect of PRDC/GFP overexpression on Bmp4 expression. Overexpression of PRDC caused a loss of Bmp4 expression that precisely overlapped with GFP/PRDC (arrows in E and F). A high power of this is shown in D' and E'. Compare region highlighted with white lines on the control side (D': unelectroporated) and electroporated (E'). (G–I) Effect of ires-GFP on Bmp7 expression. Expression of ires-GFP had no effect on endogenous Bmp7 expression (compare control side of the embryo G with H). Arrows in H and I show where GFP co-localized with the Bmp7 expression. In contrast, overexpression of PRDC caused a loss of Bmp7 expression which correlated with PRDC/GFP, compare J and K (arrowheads in K and L illustrate the GFP in the tissue and the loss of Bmp7). 1st, first pharyngeal arch; 2nd, second pharyngeal arch; 3rd, third pharyngeal arch; g, geniculate; p, petrosal.
(compare arrows in Figs. 4Q and R with the unelectroporated side P; 83% (n = 23)). Control electroporations using an ires-GFP construct resulted in no change in placode development (Delta1, 88% (n = 24); Figs. 4M–O, see arrows in N and O). Next, we examined the expression of a later neuronal marker, Phox2a, which characterizes all three placodes, the geniculate, petrosal, and nodose (Figs. 4S–X; Baker and Bronner-Fraser, 2000). Ectopic PRDC expression resulted in a reduction of Phox2a (71% (n = 21); Figs. 4V–X). For example, targeted overexpression of PRDC in the region of the geniculate (see arrows in Figs. 4W and X) resulted in a total loss of Phox2a expression when compared to the control side (arrows, Fig. 4V). Phox2a expression in the petrosal was also reduced (arrowhead in Fig. 4W). Control electroporations rarely affected endogenous Phox2a expression (70% normal (n = 10); Figs. 4S–U, compare arrows in T and U).

NeuroM is also expressed in the geniculate, petrosal, and nodose placodes (Abu-Elmagd et al, 2001), and as for Sox3, Delta1, and Phox2a, ectopic PRDC inhibited the expression of NeuroM (75% (n = 12); data not shown). Finally, we analysed the effect of PRDC on HuC, a postmitotic neuronal marker (Marusich et al, 1994; Figs. 4Y–Z). Ectopic expression of PRDC/GFP correlated with the loss of HuC (arrows in Figs. 4Z and Z′; 66% (n = 9)). Expression was much reduced on the electroporated side (asterisk in Fig. 4Z) compared to the control side of the embryo (Fig. 4Y). These results demonstrate that lowering the level of BMP signalling by overexpression of PRDC results in a loss of epibranchial neurogenic markers.

Loss of PRDC causes a local increase in Bmp4 (but not Bmp7) expression and results in larger epibranchial placodes.

To understand whether PRDC is required to define the spatial extent of the endogenous expression of Bmps we designed siRNA constructs to block expression of PRDC (siRNA/PRDC). If the role of

---

**Fig. 4.** Overexpression of PRDC inhibits epibranchial placode neurogenesis. Left panels (A, D, G, J, M, P, S, V, Y) are the control side of electroporated embryos; middle panels (B, E, H, K, N, O, T, W, Z), electroporated side; and right panels (C, F, I, L, O, R, U, X, Z′), GFP of the electroporated construct. Electroporations were targeted to the ectoderm and embryos examined for the expression of Sox3, Pax2, Phox2a, and HuC are all stage 20/21, those analysed for Delta1 are stage 17. (A–C) ires-GFP electroporations did not affect Sox3 expression (arrows in B and C); however, PRDC/GFP caused a reduction in Sox3 (D–F; arrows in E and F). (G–I) Expression of ires-GFP had no effect on endogenous Pax2 expression; see arrows in G and H. Arrows in I show the GFP expression in all three placodes. (J–L) Overexpression of PRDC did not affect Pax2 expression; compare control side J (black arrows) to electroporated side L (black arrows) with GFP in L (white arrows). (M–O) Expression of ires-GFP had no effect on endogenous Delta1 expression; see arrows in M and N, and GFP in O (white arrows). (P–R) Overexpression of PRDC/GFP resulted in a loss of Delta1 which correlated with the GFP; see arrows in Q and R. (S–U) Expression of ires-GFP resulted in no change in Phox2a expression. Arrows in T and U show the colocalization of GFP with the Phox2a. (V–X) In contrast, overexpression of PRDC/GFP caused a loss of Phox2a where there was GFP (compare arrows in W and X). (Y–Z′) HuC expression was also reduced in PRDC/GFP embryos. Expression of PRDC/GFP was seen strongly in the second pharyngeal arch and weakly in the third (Z′); this correlated with a greater loss of HuC in the petrosal than nodose (Z). 1st, first pharyngeal arch; 2nd, second pharyngeal arch; 3rd, third pharyngeal arch; g, geniculate; n, nodose; p, petrosal.
PRDC is to limit the expression of Bmp4 (and/or Bmp7), which in turn specifies the placodes, then a loss of PRDC should result in an increase in the spatial extent of expression of these genes, resulting in larger placodes. siRNA/PRDC was expressed in the presumptive endoderm of the arches at stage 11 to knockdown endogenous PRDC expression (Figs. 5A–F). Control siRNA electroporations had no effect on

---

**Fig. 5.** Loss of endogenous PRDC induces ectopic Bmp4 expression. Left panels (A, D, J) are control side of electroporated embryos; middle panels (B, E, H, K), electroporated side; and right panels (C, F, I, L), GFP of the electroporated construct. Electroporations were targeted to the endoderm and embryos were harvested at stage 20/21. (A–C) Electroporation of the control siRNA construct had no effect on endogenous PRDC expression (compare control side, A, with electroporated, B). (D–E) Electroporation of siRNA/PRDC caused a knockdown of endogenous PRDC expression (compare arrows on control side, D, with those of the electroporated side, E). D′ and E′ are high powers of the region of knockdown. Black arrows and asterisk in E′ clearly demonstrate the loss of PRDC expression. GFP was found in the endoderm of the second arch where the loss of PRDC was seen (arrowheads in F and asterisk in F′). (G–I) siRNA control electroporations had no effect on Bmp4 expression. Arrows and arrowheads in H and I demonstrate the GFP and Bmp4 expression. In contrast, overexpression of siRNA/PRDC (J–L) caused an increase in Bmp4 expression. Expression of siRNA/PRDC in the endoderm of the second arch (arrows in L) resulted in ectopic Bmp4 (arrowheads in K). 1st, first pharyngeal arch; 2nd, second pharyngeal arch; 3rd, third pharyngeal arch; g, geniculate; ov, otic vesicle; p, petrosal.
endogenous PRDC expression (100% (n = 4); Figs. 5A–C); however, siRNA/PRDC targeted to the endoderm of the arches caused a reduction in PRDC expression (60% (n = 15); compare control side to electroporated, see arrows in Figs. 5D and E). This loss of endogenous PRDC can be seen at higher power in Fig. 5E′ when compared to the control side D′ (black arrows in E′) and correlates...
with the GFP (see asterisk in Figs. 5E′ and F′). We found that reducing the endogenous level of PRDC expression caused an increase in Bmp4 expression (58% (n = 12); Figs. 5 J–L) close to the endodermal expression of siRNA/PRDC (arrows in Figs. 5K and L), whereas control siRNA electroporations were normal (92% (n = 12); Figs. 5G–I).

We also tested whether a loss of PRDC causes an increase in Bmp7 expression, as PRDC also inhibits Bmp7 (as shown by our animal cap experiments and overexpression of PRDC data). We did not see a noticeable increase in Bmp7 expression (n = 15; data not shown) suggesting that loss of PRDC is not sufficient to cause ectopic Bmp7 expression, possibly reflecting the timing of expression of these two genes, as PRDC is expressed from stage 15, whereas Bmp7 is expressed from stage 13.

Overexpression of PRDC causes a loss of Bmp4 in the placodes and a loss of epibranchial placode markers (Sox3, Delta1, Phox2α, NeuroM, and HuC). Next, we investigated whether inhibition of PRDC, leading to an increase in Bmp4, resulted in larger placodes (Fig. 6) by analysing the expression of early placode markers, Sox3; Pax2, and late markers of the placodes, Phox2α/HuC. We found that a loss of PRDC in the endoderm of the pharyngeal arches did not affect the endogenous expression of Sox3 (Figs. 6A–C; arrows in A and B; 78% of embryos normal (n = 9)) or Pax2 (Figs. 6D–F; compare arrows in D and E with GFP in F; 100% normal (n = 12); control siRNA experiments looking at Pax2 expression were also 100% normal (n = 10, data not shown)). This suggests that the early specification of the placodes occurs normally. In contrast, loss of PRDC (arrows indicate the location of the siRNA/PRDC; Fig. 5L) caused an increase in the expression of Phox2α, a neuronal placode marker, resulting in larger placodes (Figs. 6G–L; compare control electroporations in Fig. 6H; 78% normal (n = 13), and experimental, Fig. 6K, 67% with an increase in Phox2α (n = 12); arrows in Fig. 6K show the extra expression). We also analysed the expression of HuC (Figs. 6M–O) and found that loss of PRDC caused an increase in HuC expression (75% (n = 8); arrows in Fig. 6N).

In the few cases where embryos were electroporated with a control siRNA construct and displayed a change to normal expression, we saw a decrease in expression, but never an increase in placode markers. In addition, neither Sox3 nor Pax2 expression was affected, demonstrating that the increase in Phox2α/HuC expression following knockdown of PRDC was due to an increase in neurogenesis not due to a loss of tissue between them.

**Ectopic Bmp4 induces epibranchial neurons**

We have shown that overexpression of PRDC inhibits the endogenous expression of Bmp4 and Bmp7 and results in a loss of epibranchial placodes. To investigate whether PRDC does this in vivo, we knocked down PRDC and found that it only affected Bmp4 signalling and that this resulted in an increase in epibranchial neurogenesis, strengthening our hypothesis that PRDC is required to modulate the activity of BMP4 rather than BMP7. To test whether it is the small domain of Bmp4 expression that is restricting the amount of neurogenesis in the placodes, we ectopically expressed Xenopus BMP4 throughout the presumptive pharyngeal ectoderm using a BMP4-iresGFP construct and analysed the expression of Pax2 and Phox2α (Figs. 7A–F). We saw no ectopic expression of Pax2 following BMP4 overexpression (n = 12; data not shown), consistent with the siRNA data: loss of PRDC induces BMP4 locally and this does not effect Pax2 signalling. In a few cases, we observed a loss of Pax2 expression but this occurred only in embryos that were morphologically affected by the ectopic BMP4, possibly due to the well-known activity of BMP4 in inducing cell death (Graham et al., 1994).

In contrast, ectopic Phox2α expression was detected following ectopic expression of BMP4 (80% (n = 10); Figs. 7C and D), We found that the dorsal ectoderm is competent to express Phox2α in response to BMP4, and competence is not restricted to the area immediately adjacent to the placodes (arrows, Fig. 7F). Thus, the presence of a locally restricted BMP inhibitor, PRDC, confines the activity of BMP4 to a small domain and thereby restricts epibranchial placode neurogenesis.

**Discussion**

Previous research has demonstrated that formation of the epibranchial placodes initially involves a combination of FGF signalling and the repression of Wnt signalling (Nechiporuk et al., 2005, 2007; Ohyama et al., 2006; Freter et al, 2008; reviewed by McCabe and Bronner-Fraser, 2009). BMP signalling is then required from the endoderm of the pharyngeal pouches to induce neurogenesis in the overlying placodes (Fig. 8A; Begbie et al, 1999; Holzschuh et al., 2005). Here, we have investigated whether there is a later requirement for BMPs in the placodes themselves. If there were such a requirement for BMPs, then it would be expected that a BMP antagonist would be expressed locally to restrict the expression of BMPs to specific domains. To date, no known BMP inhibitors have been identified that fulfill this role (Bardot et al. 2001; Ogita et al., 2001; Gerlach-Bank et al, 2002; Müller et al., 2006; this work). We have cloned chick PRDC, a member of the Cerberus/Dan family of extracellular BMP antagonists (Sudo et al, 2004) and find that PRDC expression is restricted to the endoderm of the pharyngeal pouches (Fig. 8B). Using a combination of overexpression and knockdown approaches, we demonstrate that PRDC can modulate the expression of Bmp4 and is therefore a candidate regulator of the spatial extent of placodal neurogenesis.

Overexpression of PRDC inhibited the expression of both Bmp4 and Bmp7, but to understand the endogenous requirement for PRDC, we also inhibited its expression and analysed the effect on BMP signalling. We find that loss of the BMP antagonist PRDC resulted in a local increase in Bmp4 expression but not in Bmp7 expression. PRDC is expressed later than Bmp7 but is initially seen at the same stage as Bmp4, suggesting that PRDC is required in ovo to modulate the activity of BMP4 rather than BMP7. Thus, it may be that Bmp7 might have a totally different (as yet unknown) role at later stages and only be required for epibranchial patterning at stage 13. Another possibility is that Bmp7 is required to induce PRDC and/or BMP4 expression and therefore a loss of PRDC would have no effect on the endogenous expression of Bmp7, as Bmp7 would be upstream of PRDC.

We monitored the development of the placodes after overexpression and knockdown of PRDC. Sox3 and Pax2 are two of the earliest genes expressed in the epibranchial placodes with their expression being initially widespread but then becoming restricted to the placodes (Hidalgo-Sánchez, et al, 2000; Abu-Elmagd et al, 2001). Recent research has shown that Sox3 is essential for the neurogenic capacity of the ectoderm (Tripathi et al, 2009). However, as cells leave the placodes and undergo neurogenesis, Sox3 expression is lost (Abu-
Elmagd et al., 2001; Ishii et al., 2001). We find that after overexpression of PRDC, Pax2 expression is maintained but Sox3 expression is lost. This suggests that the early specification of the placodes in these embryos is normal but that neurogenesis is affected. Bmp7, expressed from stage 13 in the pharyngeal endoderm, is required to induce epibranchial neurogenesis. We propose that Bmp4, expressed from stage 15 in the placodes themselves, is required to maintain them; hence, if Bmp expression is lost, the initial patterning of the placodes is normal but placode neurogenesis would be inhibited. Delta1 is expressed in the earliest neurogenic cells of the placodes and its expression is restricted to cells within the placodal ectoderm. Once neurogenesis occurs, NeuroM and Phox2a are expressed in the placodes and in cells migrating away. HuC is then expressed in cells after their delamination from the placodes (Fig. 8B; Begbie et al., 1999; Abu-Elmagd et al., 2001; Begbie et al., 2002; Begbie et al., 2004). A loss of Bmp signalling caused loss of the neurogenic markers Delta1, NeuroM, Phox2a, and HuC indicating that there was a failure of maintenance of the placodes (Fig. 8C).

In contrast, loss of PRDC caused an increase in the expression of late epibranchial neurogenic markers, such as Phox2a and HuC but had no effect on early specification of the placode, as shown by normal expression of Sox3 and Pax2 (Fig. 8D). If BMP4 is required to maintain epibranchial neurogenesis (rather than the induction of the placodes), we would not expect a change in the expression of these two genes. Sox3 expression is lost once neurogenesis begins (Abu-Elmagd et al., 2001) and Pax2 is expressed in the ectoderm of the placodes rather than the neurons (Baker and Bronner-Fraser, 2000). In addition, both genes are initially expressed in the epibranchial placode region before the onset of Bmp4/7 expression, so are more likely to be upstream rather than downstream of BMP signalling.

Fig. 7. Overexpression of BMP4 induces Phox2a expression. Left panels (A, C) are the electroporated side of the embryo; and right panels (B, D), the GFP of the electroporated construct. Electroporations were targeted to the ectoderm and embryos were harvested at stage 20. (A) Expression of Phox2a after electroporation of ires-GFP. There is no change in the geniculate where GFP is expressed (see arrows, B). Following electroporation with BMP4/GFP, ectopic Phox2a expression (arrowheads in C) was detected. GFP of the BMP4/GFP construct can be seen in (D). (E) Control side of an embryo showing normal Phox2a expression in the petrosal and geniculate compared to (F) ectopic Phox2a throughout the cranial ectoderm. 1st, first pharyngeal arch; 2nd, second pharyngeal arch; g, geniculate; p, petrosal.
Changes in Bmp4 expression affected the development of neurons and their migration from the placode as indicated by the ectopic expression of both Phox2a and HuC. This activity can be directly attributed to BMP4 as overexpression of BMP4 itself has a phenotype comparable to a knockdown of PRDC (and therefore, a local increase in Bmp4 expression; Fig. 8E).

We have cloned and characterised the expression and function of chick PRDC and show that this gene is essential during development to control the amount of BMP4 expression in the epibranchial placodes. In addition, we have demonstrated an important and previously unidentified requirement for BMP4 in the specification and maintenance of the placodal neurons. In order for placodes to form as spatially discrete structures, there must be a locally expressed BMP inhibitor that restricts the expression of BMP4 to a small, specific domain. It has previously been suggested that the pharyngeal pouches can act as a source of long-range inducers and short-range inhibitors of neurogenesis in Xenopus (Schlosser, 2003). The ability of BMPs to signal at long range and the importance of extracellular inhibitors in limiting the range of such signals in other embryonic tissues has been demonstrated in several Xenopus studies (e.g. Dosch et al., 1997; Jones and Smith, 1998; Blitz et al., 2000). In line with this model, the early expression of BMP7 may constitute a long-range inducing signal that first specifies a 'neurogenic field', whereas, at later stages, long-range activity of BMP4 is kept at bay by local – possibly short-range – inhibition by PRDC, resulting in the formation of spatially discrete placodes.

Acknowledgments

We would like to thank Ali Brivanlou in whose lab the initial cloning of PRDC was performed and Richard Wingate and Ignacio Munoz-Sanjuan for their comments on the manuscript. This work was funded by the MRC. E.B. is the recipient of a MRC Career Development Fellowship (G120/782).

References


Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391, 351–357.


