Differentiation 79 (2010) 251–259

Contents lists available at ScienceDirect

Differentiation

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

Involvement of Neptune in induction of the hatching gland and neural crest in the *Xenopus* embryo

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ARTICLE INFO

Article history:
Received 9 July 2009
Received in revised form
28 December 2009
Accepted 31 January 2010

Keywords:
Xenopus
Hatching gland
Neural crest
pax3
KLF

ABSTRACT

Neptune, a Krüppel-like transcription factor, is expressed in various regions of the developing *Xenopus* embryo and it has multiple functions in the process of development in various organs. *In situ* hybridization analysis showed that Neptune is expressed in the boundary region between neural and non-neural tissues at the neurula stage, but little is known about the function of Neptune in this region. Here, we examined the expression and function of Neptune in the neural plate border (NPB) in the *Xenopus* embryo. Depletion of Neptune protein in developing embryos by using antisense MO caused loss of the hatching gland and otic vesicle as well as malformation of neural crest-derived cranial cartilages and melanocytes. Neptune MO also suppressed the expression of hatching gland and neural crest markers such as *he*, *snail2*, *sox9* and *msx1* at the neurula stage. Subsequent experiments showed that Neptune is necessary and sufficient for the differentiation of hatching gland cells and that it is located downstream of pax3 in the signal regulating the differentiation of these cells. Thus, Neptune is a new member of hatching gland specifier and plays a physiological role in determination and specification of multiple lineages derived from the NPB region.

1. Introduction

In anuran amphibian embryos, the boundary region between the neural plate and the epidermis comprises three distinct cell lineages: the neural crest (NC), the hatching gland and the placodal ectoderm (Hong and Saint-Jeannet, 2007). As the neural tube closes, the NC cells delaminate from the dorsal neural tube and migrate to various parts of the embryo, where they differentiate into a wide range of cell types, including neurons, glia of the peripheral nervous system, smooth muscle cells, craniofacial cartilages, melanocytes, bones, endocrine cells and fin (Mayor and Aybar, 2001; Le Douarin et al., 2004). Extensive studies using various vertebrate animals, including mice, chickens, frogs and zebrafish, have shown that interaction between the neural and non-neural ectoderms, as well as the signals derived from the underlying paraxial mesoderm, are involved in specification of the NC in embryos (Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Bonstein et al., 1998). At the molecular level, diffusible molecules such as BMP, Wnt, FGF and retinoic acid play important roles in NC induction (Mancilla and Mayor, 1996; Mayor et al., 1997; Marchant et al., 1998; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Garcia-Castro et al., 2002; Monsoro-Burq et al., 2003). At first, a gradient of BMP signaling established by BMPs and BMP antagonists is involved in the specification of fates of ectodermal cells. A high level of BMP specifies the epidermis and a low level of BMP specifies the neural plate. An intermediate level of BMP determines the prospective NC-forming region (referred to as neural plate border, NPB) in the ectoderm. Subsequently, the ectoderm at the NPB becomes competent to respond to transforming signals, such as signals from Wnt, FGF and retinoic acid, which specify and maintain the NC (Villanueva et al., 2002; Aybar and Mayor, 2002). Once the position of the NPB is determined by BMP, Wnt and FGF signals, a group of transcription factors (referred to as NPB specifiers), such as zic, msx, pax and dlx, are expressed in the broad area between the neural and non-neural regions (Meulemans and Bronner-Fraser, 2004). These factors determine the expression domain of another set of transcription factors (NC specifiers), such as snail and soxE, in the restricted region of the NPB.

Hatching gland cells arise from the superficial layer of the ectoderm and are located dorsal to the NC-forming region, and they produce enzymes that digest the fertilization envelope and jelly coat (Drysdale and Elinson, 1991). The same combination of signals, such as BMP and Wnt, are sufficient for induction of NC cells and hatching gland cells (McGrew et al., 1999), suggesting that the differentiation of hatching gland cells is also controlled by NPB specifiers described above. Further studies on the molecular dissection of NPB specifiers revealed that cooperation

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doi:10.1016/j.diff.2010.01.003

of pax3 and zic1 is involved in the early specification of NC cells (Hong and Saint-Jeannet, 2007; Sato et al., 2005; Monsoro-Burq et al., 2005) as well as hatching gland cells (Hong and Saint-Jeannet, 2007). It has also been shown that msx1/2 has an essential role in the initiation of NPB specification (Monsoro-Burq et al., 2005; Tribulo et al., 2003; Khadka et al., 2006) and that it controls the number of NC cells (Tribulo et al., 2004). Thus, it is likely that each NPB specifier molecule has its own role for determining the fate of NC cells and hatching gland cells. The interactions of these factors, however, are complex and the hierarchical relationship has not been fully elucidated.

Neptune was first identified as a Krüppel-like transcription factor related to zebrafish biklf (blood island-enriched Krüppel-like factor) in *Xenopus*. It has been shown that Neptune is expressed in ventral blood islands at the tailbud stage and is involved in erythrocyte differentiation (Kawahara and Dawid, 2000; Kawahara and Dawid, 2001; Huber et al., 2001). More recently, we found that Neptune has a role in posterior axis formation through FGF signaling (Takeda et al., 2005). In the course of studies on Neptune in mesoderm lineages, we found that *Neptune* is temporarily expressed in the NPB region at the neurula stage of the *Xenopus* embryo. Since the expression pattern of *Neptune* is very similar to that of NPB specifiers such as msx1 and pax3, we attempted to determine its novel function in NC specification. In the present study, we utilized the Morpholino (MO) strategy to knockdown Neptune expression. Injection of MO into ectodermal cells caused severe damage in the hatching gland, NC and otic vesicle, and overexpression of Neptune induced the ectopic expression of *he*, a hatching enzyme marker, at the injected side of embryo. The present study demonstrated that Neptune is one of the essential factors controlling early specification of the NPB in the *Xenopus* embryo.

2. Materials and methods

2.1. Embryonic manipulation, mRNA synthesis, morpholino and microinjection

Xenopus laevis embryos were obtained as previously described (Takeda et al., 2005) and developmental stages were determined according to Nieuwkoop and Faber (1994). Capped mRNAs were synthesized using a MEGAscript kit (Ambion). For glucocorticoid receptor-fusion *Neptune* construction (*Neptune-GR*), the coding sequence of the hormone-binding domain derived from *Xbra-GR* (Tada et al., 1997) was fused to the 3'-end of the Neptune-coding sequence and subcloned into pCS2+. *Neptune-GR* lacking the 5'UTR sequence (*Δ5'UTR-Neptune-GR*) was also made by the PCR strategy and subcloned into pCS2+. These plasmids were linearized with Not I and their RNAs were synthesized by SP6 polymerase. *pax3* in pBluescript was linearized by Not I and sense RNA was synthesized by T7 polymerase. The Neptune antisense morpholino oligonucleotide (NepMO) was designed complementary to the sequence corresponding the translation start site and the 5'-UTR (nucleotides −17 to +8, 5'-CTGGGGAGCACGTTAG-GATGAGTGT-3'). A mutated NepMO (5misMO) (targeting the same region with 5 mutations as follows: 5'-CTGGGCAGGACCT-TAGCATCAGTGT-3') and the standard control morpholino (CoMO) supplied by the manufacturer (Gene Tools) were used as negative controls. Microinjection was performed according to the method described previously (Takeda et al., 2005). RNA or MO was coinjected with *β-galactosidase* mRNA (*β-gal*) as a lineage tracer into 2- or 4-cell-stage embryos using a Nanoject injector (Drummond). Red-Gal (Research Organics) was used for the substrate of *β*-gal. To activate Neptune-GR in a hormone-dependent manner, dexamethasone (DEX; final concentration of

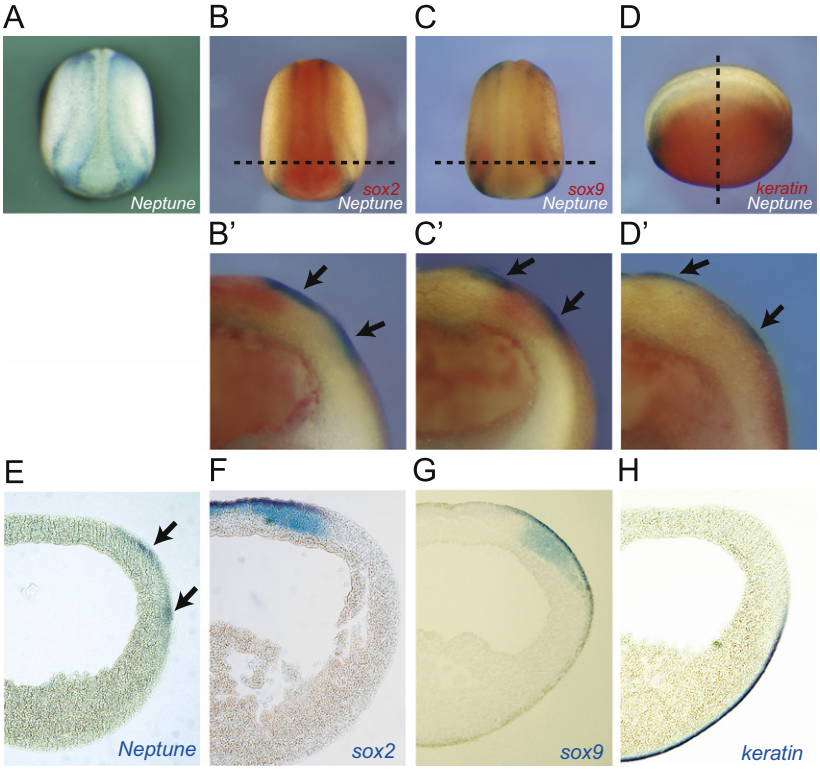

Fig. 1. Expression pattern of *Neptune* at the neurula stage determined by whole-mount *in situ* hybridization analysis. (A) *Neptune* is expressed at the border region of the neural plate at stage 15 (n=12). Two lines of positive area along the neural fold were characteristic of the neurula stage. (B–D) Double *in situ* hybridization of *Neptune* (BM purple) with *sox2* (Fast-Red) (B and B', n=10), *Neptune* with *sox9* (C and C', n=10) and *Neptune* with *keratin* (D and D', n=10). Dissection of stained embryos at the levels indicated by the broken lines in B–D showed the expression of *Neptune* (arrows) at the boundary between *sox2*- and *sox9*-positive regions and at the boundary between *sox9*- and *keratin*-positive regions (B'–D'). (E–H) Cross-sections of stained embryos. (A–C) Dorsal view and anterior to the bottom. (D) Lateral view and anterior to the left. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)