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Conserved expression of mouse *Six1* in the pre-placodal region (PPR) and identification of an enhancer for the rostral PPR

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ABSTRACT

All cranial sensory organs and sensory neurons of vertebrates develop from cranial placodes. In chick, amphibians and zebrafish, all placodes originate from a common precursor domain, the pre-placodal region (PPR), marked by the expression of *Six1/4* and *Eya1/2*. However, the PPR has never been described in mammals and the mechanism involved in the formation of PPR is poorly defined. Here, we report the expression of *Six1* in the horseshoe-shaped mouse ectoderm surrounding the anterior neural plate in a pattern broadly similar to that of non-mammalian vertebrates. To elucidate the identity of *Six1*-positive mouse ectoderm, we searched for enhancers responsible for *Six1* expression by *in vivo* enhancer assays. One conserved non-coding sequence, Six1-14, showed specific enhancer activity in the rostral PPR of chick and *Xenopus* and in the mouse ectoderm. These results strongly suggest the presence of PPR in mouse and that it is conserved in vertebrates. Moreover, we show the importance of the homeodomain protein-binding sites of Six1-14, the *Six1* rostral PPR enhancer, for enhancer activity, and that Dlx5, Msx1 and Pax7 are candidate binding factors that regulate the level and area of *Six1* expression, and thereby the location of the PPR. Our findings provide critical information and tools to elucidate the molecular mechanism of early sensory development and have implications for the development of sensory precursor/stem cells.
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Introduction

In vertebrates, all cranial sensory organs (except the retina) and sensory neurons originate from the cranial placodes (Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005; Schlosser, 2006; Streit, 2004). Adenohypophyseal, olfactory, lens, trigeminal, otic and epibranchial placodes have been described in amniotes, which give rise to adenohypophysis, olfactory epithelium, lens, trigeminal ganglion, inner ear and vestibulo-acoustic ganglion, and epibranchial sensory ganglia (geniculate, nodose and petrosal ganglia), respectively. All placodes share similar characteristics; they form columnar epithelia adjacent to the neural tube, some of the cells delaminate to form sensory ganglia (Graham et al., 2007), and they are neurogenic with the exception of lens and adenohypophyseal placodes. Moreover, there is growing evidence supporting the notion that all placodes originate from a common precursor domain termed the pre-placodal region (PPR) or pan-placodal primordium (Baker and Bronner-Fraser, 2001; McLarren et al., 2003; Schlosser and Ahrens, 2004; Streit, 2002). The horseshoe-shaped domain encircling the anterior neural plate was initially reported to be competent in forming multiple placodes in amphibians (Jacobson, 1963). Fate mapping in chick and zebrafish revealed overlapping distribution of precursor cells of different placodes in this region (Bhattacharyya et al., 2004; Kozlowski et al., 1997; Streit, 2002, Xu et al., 2008). Identification of *Six* (*Six1* and *Six4*) and *Eya* (*Eya1* and *Eya2*) genes (Esteve and Bovolenta, 1999; Ishihara et al., 2008a; Litsiou et al., 2005; Sahly et al., 1999; Schlosser and Ahrens, 2004; Streit, 2002) as specific markers whose expression match the PPR defined by fate mapping provided further evidence that PPR is a territory with a distinct molecular signature. In addition, there is evidence that cells in the PPR share the property of expressing *Pax6* followed by additional lens-marker genes and form the lens when cultured in isolation (Bailey et al., 2006).

Analyses of mutants and morphants in the mouse and zebrafish have confirmed the role of *Six1* and *Eya1* in sense organ development. Severe defects are found in multiple sensory organs or placode derivatives; the anterior pituitary (adenohypophysis), olfactory epithelium, trigeminal ganglion, inner ear and epibranchial ganglia (Bricaud and Collazo, 2006; Chen et al., 2009; Ikeda et al., 2007; Konishi et al., 2006; Kozlowski et al., 2005; Laclef et al., 2003; Li et al., 2003; Nica et al., 2006; Ozaki et al., 2004; Xu et al., 1999; Zheng et al., 2003; Zou et al., 2004). In human, mutations of *SIX1* and *EYA1* cause a sensory disorder called branchio-oto-renal syndrome (Ruf et al., 2004). Importantly, these genes have specific functions in the PPR

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prior to the specification of individual placodes and/or determine characteristics shared among placodes. Overexpression of *Six1* and *Eya1* expanded the PPR at the expense of neural crest and epidermis (Brugmann et al., 2004; Christophorou et al., 2009). Both genes support placodal neuronal progenitor proliferation and subsequent neuronal differentiation through their effects on *SoxB1* expression (Schlosser et al., 2008). Finally, the naïve ectoderm only becomes competent to respond to otic placode-inducing signals when it has first adopted a pre-placodal identity characterized by the expression of *Eya2* (Martin and Groves, 2006). Thus, there is a general agreement that the PPR seems to represent a pool of sensory precursor cells and a regulatory network consisting of various genes including *Six1/4* and *Eya1/2* confers pre-placodal characteristics to the region (Baker and Bronner-Fraser, 2001; Christophorou et al., 2009; Ohyama et al., 2007; Schlosser, 2006). Recent studies have identified several signaling molecules involved in the induction of the PPR (Ahrens and Schlosser, 2005; Brugmann et al., 2004; Esterberg and Fritz, 2009; Glavic et al., 2004; Hong and Saint-Jeannet, 2007; Litsiou et al., 2005; Sjodal et al., 2007; Solomon and Fritz, 2002; Woda et al., 2003). However, three important questions remain unanswered: 1) Does the PPR exist in mammals? 2) Is there any specific enhancer that can be used to label a part of or the entire PPR? and 3) If such an enhancer really exists, what is the regulatory mechanism activating such a PPR-specific enhancer?

Identification of major enhancers that control the expression of a key developmental gene in a given developmental processes is critical for elucidating its underlying molecular basis, as shown in the case of *Sox2* regulation during neural induction (Papanayotou et al., 2008; Takemoto et al., 2006; Uchikawa et al., 2003). Elucidation of the regulatory mechanisms of *Six1/4* and *Eya1/2* expression could provide a clue to answer the above questions. Unfortunately, none of the major conserved enhancers of *Eya1* activates transcription in the PPR (Ishihara et al., 2008b). Also, the lack of information as to whether *Six4* and *Eya2* play a conserved critical role during early sensory development points to the importance of analyzing *Six1* regulation. In the mouse, *Six1* expression is detected in all placodes (excluding the lens placode) and their derivatives (Gu et al., 2004; Laclef et al., 2003; Oliver et al., 1995; Ozaki et al., 2004), and appears at embryonic day 7.5 (E7.5) in the endoderm (Gu et al., 2004) and at E8.0 in the rostral region of the embryo (Chen et al., 2009). However, its expression in the horseshoe-shaped PPR or even the presence of such region in mouse embryo remains elusive.

Here, we report mouse *Six1* expression in the ectoderm surrounding the anterior neural plate in a pattern essentially similar to that of non-mammalian vertebrates. To elucidate the identity of *Six1*-positive ectoderm, we found that one conserved sequence had specific enhancer activity in the PPR of chick and *Xenopus* and in the ectoderm of mouse. Together, the results suggest the presence of a PPR in mouse. We also analyzed the regulatory mechanism activating the unique *Six1* PPR enhancer and identified how *Six1*-positive domain/PPR is established.

Materials and methods

Genomic sequence analysis

The genomic sequences covering *Six1* were obtained from Ensembl. Global pairwise alignment was carried out using shuffle-LAGAN (Brudno et al., 2003), and the results were visualized using the VISTA Browser (Frazer et al., 2004). Conserved transcription factor binding sites were identified using rVISTA (Loots and Ovcharenko, 2004), Mulan (Ovcharenko et al., 2005) or TESS (Schug, 2008).

Reporter plasmid and transgene construction

The conserved non-coding sequences (CNSs) were isolated by PCR from genomic DNAs or by digesting genomic subclones and ligated into ptkEGFP (Uchikawa et al., 2003). mSix1-14 (mouse Six1-14, 565 bp) and cSix1-14PCR (chick Six1-14, 784 bp) were isolated using the primers listed in Table S1. The ptkmRFP1ver2 (Inoue et al., 2007) was used for construction of mRFP1 and multimerized reporters. Mutated ptkmRFP1-mSix1-14 reporters were constructed using the primers listed in Table S2. For mouse transgenesis, wild-type and mutated Six1-14 were each ligated into ASShsp68lacZpA (Sasaki and Hogan, 1996) or ASStkintronlacZpA and transgene DNA fragments were excised and purified using QIAEX II (Qiagen, Hilden, Germany). For *Xenopus* transgenesis, wild-type mSix1-14 was ligated into ISpBSIISK + betaGFP (Ogino et al., 2006). All plasmids were verified by DNA sequencing and purified by QIAfilter or EndoFree Plasmid Kit (Qiagen). Table S3 shows a list of plasmids.

Animals

Mice were housed in an environmentally-controlled room in the CDB, RIKEN Kobe and in the Center for Experimental Medicine of Jichi Medical University, under the guidelines for animal experiments. Fertilized eggs of chick were purchased from Shiroyama Poultry Farm (Kanagawa, Japan), and incubated at 38 °C in a humidified rocking incubator. The developmental stage of chick embryos was determined according to Hamburger and Hamilton (1951). *Xenopus* were kept in the animal facility at NAIST. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Jichi Medical University.

Electroporation into chick embryos and detection of enhancer activity

Electroporation into chick embryos (Fig. 3A) was performed as described previously (Ishihara et al., 2008b). In the initial screening of enhancers, electroporation was verified using pCAG-HcRed (Matsuda and Cepko, 2004) that drives ubiquitous expression of HcRed under the control of the strong CAG promoter/enhancer (Figs. 3B,C). For mSix1-14 mutation analysis, the ratio of the amount of plasmid for mRFP1 reporters to EGFP control was kept constant (mRFP: EGFP = 1:2) to adjust fluorescence intensity, and mRFP1 and EGFP images were taken at the same exposure time. Embryos were examined at 6, 12 and 24 h post-electroporation (h.p.e.). We performed electroporation until we obtained more than 5 embryos with homogeneous DNA distribution and normal morphology. The patterns of enhancer activities were highly reproducible, and essentially the same results were obtained from those embryos. For histological analysis, embryos were fixed and cryosections (14 μm) were prepared.

Generation and analysis of transgenic Xenopus embryos

Transgenic *Xenopus* embryos were generated using the modified sperm nuclear transplantation method (Ogino et al., 2008). Expression of EGFP mRNA was detected by *in situ* hybridization for maximum sensitivity. Stained embryos were fixed, embedded in 2% agarose and thick vibratome sections (100 μm) were prepared.

Generation and histological analysis of transgenic mice

Transgenic mice were generated by microinjection using fertilized eggs of CD-1 (ICR) using a standard protocol (Nagy et al., 2003). For genotyping E10.5 embryos, yolk sac DNA was isolated and subjected to PCR with primers mSix1-14-1/mhsp68R or mSix1-14-1/ptkEGFP-RP (Table S4). Embryos at E8.0 were first fixed and processed for X-gal staining. Whole-embryo DNAs from lacZ-negative embryos were genotyped using the aforementioned primers and primers specific to lacZ (genotyping) and *Six1* (to monitor DNA quality) (Table S4). Mouse embryos were fixed and processed for X-gal staining as

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