Developmental Biology 336 (2009) 327–336

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Genomes & Developmental Control

Activation of Six1 target genes is required for sensory placode formation

Nicolas A.D. Christophorou, Andrew P. Bailey [1], Sven Hanson, Andrea Streit *

Department of Craniofacial Development, King's College London, Guy's Campus, Tower Wing Floor 27, London SE1 8RT, UK

ARTICLE INFO

Article history:
Received for publication 12 June 2009
Revised 16 September 2009
Accepted 16 September 2009
Available online 23 September 2009

Keywords:
Chick
Eyes absent
Ear
Eye
Lens
Olfactory epithelium
Pax2
Pax3
Pax6
Sine oculis

ABSTRACT

In vertebrates, cranial placodes form crucial parts of the sensory nervous system in the head. All cranial placodes arise from a common territory, the preplacodal region, and are identified by the expression of Six1/4 and Eya1/2 genes, which control different aspects of sensory development in invertebrates as well as vertebrates. While So and Eya can induce ectopic eyes in *Drosophila*, the ability of their vertebrate homologues to induce placodes in non-placodal ectoderm has not been explored. Here we show that Six1 and Eya2 are involved in ectodermal patterning and cooperate to induce preplacodal gene expression, while repressing neural plate and neural crest fates. However, they are not sufficient to induce ectopic sensory placodes in future epidermis. Activation of Six1 target genes is required for expression of preplacodal genes, for normal placode morphology and for placode-specific Pax protein expression. These findings suggest that unlike in the fly where the Pax6 homologue Eyeless acts upstream of Six and Eya, the regulatory relationships between these genes are reversed in early vertebrate placode development.

Introduction

In *Drosophila*, *sine oculis* (*so*) and *eyes absent* (*eya*) are nuclear factors that play a key role during compound eye development. Together with seven other transcriptional regulators they form the retinal determination (RD) network, a complex gene regulatory network that controls photoreceptor cell specification in the eye-antennal disc (reviewed in Treisman, 1999; Kumar and Moses, 2001; Donner and Maas, 2004; Pappu and Mardon, 2004). These genes have been placed into a functional network because of their overlapping expression patterns (Bessa et al., 2002) as well as results of loss- and gain-of-function experiments (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994; Serikaku and O'Tousa, 1994; Halder et al., 1995; Bonini et al., 1997; Shen and Mardon, 1997; Czerny et al., 1999; Seimiya and Gehring, 2000; Weasner et al., 2007) and biochemical data (Chen et al., 1997; Pignoni et al., 1997; Czerny et al., 1999; Niimi et al., 1999; Punzo et al., 2002; Ostrin et al., 2006). Like other members of the RD network, So and Eya function is required for normal eye formation and they have the unique ability to induce ectopic eyes when misexpressed in non-retinal tissue and appear to act synergistically (Bonini et al., 1997; Pignoni et al., 1997; Seimiya and Gehring, 2000; Salzer and Kumar, 2009).

In the eye-antennal disc, *so* and *eya* are downstream of Eyeless (Ey) and Twin of Eyeless (Toy) (Halder et al., 1998; Niimi et al., 1999; Bui et al., 2000; Punzo et al., 2002); they act as a complex to activate downstream target genes and positively feed back on *ey* expression itself (Pignoni et al., 1997). As in the fly, vertebrate homologues of the RD network play an important role in eye development but in addition control different aspects of ear, olfactory and sensory ganglia formation including neurogenesis and proliferation (reviewed in Kawakami et al., 2000; Wawersik and Maas, 2000; Hanson, 2001; Donner and Maas, 2004; Silver and Rebay, 2005; Kumar, 2009). They are therefore considered to be key players in controlling cell fate determination in the cranial sensory nervous system, although their regulatory relationship is not always similar to that described in *Drosophila* (for detailed discussion see: Donner and Maas, 2004; Kumar, 2009). In vertebrates, the cranial sensory nervous system largely arises from specialised epithelia, the sensory placodes (reviewed in Baker and Bronner-Fraser, 2001; Streit, 2004; Schlosser, 2006; Streit, 2007). Even before these become morphologically distinct, *so* and *eya* homologues (*Six1* and *-4*; *Eya1* and *-2*) identify most, if not all, placode progenitor cells in what has been called the preplacodal region (PPR) located next to the anterior neural plate (Mishima and Tomarev, 1998; Esteve and Bovolenta, 1999; Sahly et al., 1999; Kobayashi et al., 2000; Pandur and Moody, 2000; Streit, 2002; McLarren et al., 2003; Bessarab et al., 2004; Bhattacharyya et al., 2004; Schlosser and Ahrens, 2004; Litsiou et al., 2005; Xu et al., 2008). Interestingly, their expression begins before that of the *ey* homolog *Pax6* or of any other member of the *Pax* gene family later found in

* Corresponding author.
E-mail address: andrea.streit@kcl.ac.uk (A. Streit).
[1] Present address: NIMR, Developmental Neurobiology, Mill Hill, London NW7 1AA, UK.

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doi:10.1016/j.ydbio.2009.09.025

different placodes (Pax2: epibranchial and otic; Pax3: trigeminal; Pax6: lens and olfactory; Li et al., 1994; Stark et al., 1997; Groves and Bronner-Fraser, 2000; Bhattacharyya et al., 2004). These observations suggest that Six and Eya proteins may play a crucial role during early sensory progenitor specification and, unlike in *Drosophila*, may act upstream of Pax genes.

Indeed, a recent study in *Xenopus* revealed that Six1 function is required for cells to acquire preplacodal character and that its misexpression leads to upregulation of genes specific for placode precursors at the expense of neural crest and epidermis (Brugmann et al., 2004). Likewise, in Six1 and -4 compound mutant mice the olfactory placode does not form (Chen et al., 2009), consistent with an early, synergistic function of both genes before placode formation. However, it has not yet been tested whether Six and Eya proteins are at the top of the genetic cascade that controls sensory fates by regulating the onset of placode-specific Pax gene expression or whether they can induce ectopic placodes, as may be expected from their eye-inducing ability in the fly. The Six family member *Six3* is expressed early in the lens territory, regulates *Pax6* (Liu et al., 2006) and has been shown to induce ectopic lens-like structures in fish (Oliver et al., 1996). However, this lens-inducing ability is confined to the PPR suggesting that this territory possesses special properties distinct from the remaining non-neural ectoderm.

In support of this idea, recent evidence shows that the acquisition of preplacodal character is an essential step for placode induction: only preplacodal cells are competent to form placodes in response to the appropriate inducing signals (Martin and Groves, 2006). Furthermore, all cells within the placode territory have a common developmental potential: irrespective of their later fate they are initially specified as lens and lens formation must be suppressed for other neurogenic placodes to develop (Bailey et al., 2006). Together, these observations suggest that the PPR has unique properties and that Six and Eya proteins may play an important role for the acquisition of PPR character.

Here we address the question of whether Six1 and Eya2 are sufficient to confer PPR properties to non-placodal cells, whether they are sufficient to induce ectopic placodes and whether they act upstream of placode-specific Pax gene expression. We show that in chick, as in the fly, Six1 and Eya2 act synergistically: together they promote preplacodal gene expression while suppressing neural and neural crest cell fates. While activation of Six1 target genes is required for the specification of placode progenitors, Six1 and Eya2 are not sufficient to impart preplacodal properties (placode competence or lens specification) to cells that normally do not contribute to the sensory nervous system. Likewise, combined expression of Six1 and Eya2 does not induce ectopic placodes. However, unlike in the fly, Six1 appears to act upstream of placode-specific *Pax* gene expression: activation of Six1 target genes is required for their expression and for ectodermal cells to acquire placode morphology.

Materials and methods

Expression constructs and morpholinos

The coding region of human Six1 (Boucher et al., 1996) and chick Eya2 (Mishima and Tomarev, 1998) were cloned into the pCAB-IRES-GFP (Niwa et al., 1991). Engrailed-Six1HD in pCS2 was a kind gift from Dr Sally Moody (Brugmann et al., 2004). Fluorescein-coupled morpholinos leading to deletion of exon3 or exon6 of chick Eya2 and respective control morpholinos were described previously (Mende et al., 2008).

Embryo culture, electroporation and explant cultures

Fertile hens' eggs (Henry Stewart) were incubated at 38 °C to reach stage HH3$^+$/4 or 5–6 (Hamburger and Hamilton, 1951) and cultured according to a modified version of New's method (New, 1955; Stern and Ireland, 1981). Electroporation of expression constructs at HH3$^+$/4 and morpholinos at HH5–6 was conducted as previously described (McLarren et al., 2003; Mende et al., 2008). After overnight culture, embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 15 min for immunohistochemistry or overnight at 4 °C for in situ hybridisation.

To assay the ability of Six1 and Eya2 to confer lens specification and FGF2 responsiveness to non-placodal cells, electroporated area pellucida or area opaca epiblast visualised by GFP fluorescence was dissected after 12 h using tungsten needles. The placode territory without underlying mesoderm and endoderm was dissected from stage HH6 embryos as positive control. Explants were kept in Tyrode's saline on ice, until collagen cultures were set up as previously described (Bailey et al., 2006). Explants were grown in vitro at 37 °C in the presence of FGF2 (250 ng/ml; R&D systems; Martin and Groves, 2006) for 20 h to assay otic marker expression or absence of any growth factors for 60–72 h to assay lens specification. Explants were then fixed for 15 min in 4% PFA in PBS for immunohistochemistry.

Whole-mount in situ hybridisation and immunocytochemistry

Whole-mount in situ hybridisation was performed using digoxigenin (DIG)-labelled antisense RNA probes as previously described (Streit et al., 1998). The following plasmids were used to generate DIG-labelled antisense riboprobes: Dlx5 (McLarren et al., 2003), Eya2 (Mishima and Tomarev, 1998), Gata3 (Sheng and Stern, 1999), GnRH1 (a gift from Dr Ian Dunn), Pax2 (a gift from Dr Martyn Goulding), Pax6 (Li et al., 1994), Pax7 (Basch et al., 2006), Raldh3 (Blentic et al., 2003), Six1 (a gift from Dr Guillermo Oliver), Six4 (Esteve and Bovolenta, 1999) and Sox2 and Sox3 (Uwanogho et al., 1995).

Immunocytochemistry for GFP was performed using a polyclonal anti-GFP antibody (Molecular Probes; 1:2000 in PBS containing 5% sheep serum, 1% Triton X-100) followed by an HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 1:500 in PBS containing 5% sheep serum, 1% Triton X-100). Samples were then processed for cryosectioning.

Immunocytochemistry on cryosections was performed using polyclonal antibodies against chick δ-crystallin (a gift from Dr J. Piatigorsky), GFP (Molecular probes) and chick Pax2 (Zymed), and monoclonal antibodies against phospho-Histone H3 (Imgenex), Pax3 and Pax6 (Developmental Hybridoma Bank). The appropriate Alexafluor 488- and 594-coupled secondary antibodies were purchased from Molecular Probes; nuclei were stained by DAPI (Molecular Probes).

To quantify the number of morpholino or GFP carrying cells expressing Pax2, Pax6 and Pax3 cells in each embryo, digital images from each section were taken after immunostaining using a Leica TCS SP5 confocal microscope. For each section, the total number of morpholino or GFP carrying cells was determined by counting the green fluorescent cells with visible nuclei (MO or GFP/DAPI$^+$). The number of Pax2$^+$, Pax3$^+$ and Pax6$^+$ cells among the morpholino carrying cells was determined by counting the MO or GFP/Pax2, -3 or -6/DAPI+ cells. An unpaired *t*-test was performed to determine the statistical significance between splice-blocking morpholinos and control morpholinos and between engrailed-Six1HD and GFP electroporated cells.

Results

Six1 and Eya2 promote preplacodal gene expression but are not sufficient to induce ectopic placodes

To assess whether Six and Eya proteins alone or in combination are sufficient to induce ectopic sensory placodes, as may be expected from their eye-inducing ability in *Drosophila*, or to impart preplacodal