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Directional cell movements downstream of Gbx2 and Otx2 control the assembly of sensory placodes

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ABSTRACT

Cranial placodes contribute to sensory structures including the inner ear, the lens and olfactory epithelium and the neurons of the cranial sensory ganglia. At neurula stages, placode precursors are interspersed in the ectoderm surrounding the anterior neural plate before segregating into distinct placodes by as yet unknown mechanisms. Here, we perform live imaging to follow placode progenitors as they aggregate to form the lens and otic placodes. We find that while placode progenitors move with the same speed as their non-placodal neighbours, they exhibit increased persistence and directionality and these properties are required to assemble morphological placodes. Furthermore, we demonstrate that these factors are components of the transcriptional networks that coordinate placode cell behaviour including their directional movements. Together with previous work, our results support a dual role for Otx and Gbx transcription factors in both the early patterning of the neural plate border and the later segregation of its derivatives into distinct placodes.

KEY WORDS: Cell migration, Lens, Live imaging, Morphogenesis, Otic, Peripheral nervous system

INTRODUCTION

Vertebrate cranial placodes give rise to crucial parts of the sensory nervous system including the olfactory epithelium, the inner ear and the sensory neurons of the cranial ganglia, as well as the lens (Schlosser, 2006; Streit, 2008). Initially, placode precursors occupy a unique territory, the pre-placodal region (PPR), where cells of different fates are interspersed (Bhattacharyya et al., 2004; Kozlowski et al., 1997; McCarroll et al., 2012; Streit, 2002; Xu et al., 2008; Pieper et al., 2011). Although it is controversial whether cell sorting segregates placode progenitors (Pieper et al., 2011), at later stages placodal cells must somehow coalesce to form spatially distinct placodes along the anterior-posterior axis (Breau and Schneider-Maunoury, 2014). In chick, Dil labelling reveals some movement of cell groups during otic, olfactory and lens placode formation (Bhattacharyya et al., 2004; Streit, 2002); while in zebrafish, cells move directionally in an integrin-α5 dependent manner as they are recruited into the otic placode (Bhat and Riley, 2011). Likewise, Xenopus laevis pre-placodal cells in the epibranchial region move directionally in response to the migration of adjacent neural crest cells (Theveneau et al., 2013). Whether or not these observed movements are a passive response to the morphogenesis of surrounding tissues, or directional movement as a consequence of cellular activities within the ectoderm itself, remains to be determined.

The transcription factor Gbx2 is required for otic specification, whereas Otx2 is needed for trigeminal, lens and olfactory specification (Steventon et al., 2012). Since both genes continue to be expressed as placodes are assembled they may mediate the coalescence of placode precursors (Hidalgo-Sánchez et al., 2000; Ogino et al., 2007; Tour et al., 2001). Therefore, we sought to repress Gbx2 and Otx2 targets in a spatially and temporally controlled manner to assess their role in the formation of otic and lens placodes. Using Xenopus, we show that whilst all cells within the deep ectoderm move at a similar velocity, placodal cells migrate with increased persistence to coalesce into distinct placodes, which in turn depend on Gbx2 and Otx2 downstream targets.

RESULTS AND DISCUSSION

Time-lapse imaging reveals the gradual emergence of sensory placodes

Within the PPR of Xenopus laevis, the deep layer of the embryonic ectoderm contributes to the sensory placodes, while the superficial layer generates an epithelium that protects the embryo from the external environment (Chalmers et al., 2002). To visualize placode cell movements, we used a grafting approach to label the deep ectoderm specifically (Fig. 1A). Donor embryos were injected with mRNA encoding nuclear RFP (nRFP) alone or together with membrane GFP (mGFP) into both blastomeres at the two-cell stage. At stage 13, a region slightly larger than the PPR (compare grafted region in Fig. 1E with the expression of the PPR marker Eya1 in a stage-matched embryo in Fig. 1F) was grafted into the same position of an unlabelled stage 13 host. At stage 16, the labelled superficial layer was removed and the un-labelled superficial ectoderm was allowed to heal (Fig. 1A). Sagittal sections through the otic region of embryos grafted with nRFP/mGFP injected PPR (at the levels indicated in Fig. 1G,H,J) show how the 2-3-cell deep ectoderm at the 18-somite stage (Fig. 1B) aggregates into a multi-layered cluster (Fig. 1C; 20-somite stage), before forming the otic vesicle by stage 28 (Fig. 1D). We performed time-lapse microscopy of embryos grafted with nRFP labelled PPR and segmented regions of coherent cells using the surface function of the image analysis tool Imaris (Fig. 1L-O; Movie 2). This analysis reveals a progressive subdivision from a homogeneous sheet of cells into regions of clustered nuclei that have a brighter fluorescence signal than the surrounding cells (Fig. 1G-O; Movie 1). As shown in Fig. 1B-D, placodes form as multi-layered aggregates of cells that are surrounded by a thin layer of non-placodal cells, therefore
showing a brighter level of fluorescence when viewed in the whole embryo (Fig. 1G-J). These cell clusters match the shape and position of placodes closely, as assessed by the expression of the posterior placode marker Eya1 and the lens marker FoxE3 in stage-matched embryos (Fig. 1K,P). The profundal/trigeminal and lateral line cells occupy a coherent domain that cannot be visually separated from surrounding non-placodal cells (Fig. 1O).

**Gbx2 and Otx2 targets are required for otic and lens placode assembly**

The convergence of placode progenitors into morphological placodes as observed by our time-lapse analysis is mirrored by changes in Gbx2 and Otx2 expression: they are broadly expressed at neurula stages (Schlosser and Ahrens, 2004; Steventon et al., 2012) and continue to be expressed in the otic and lens placode at later stages (Fig. 2A-D) (Hidalgo-Sánchez et al., 2000; Ogino et al., 2007; Tour et al., 2001). The latter coincides with the expression of placode-specific genes like Pax2 (Fig. 2E,F) in the otic domain and Foxe3 in the lens (Fig. 2G,H). We therefore asked whether Gbx2 and Otx2 mediate placode assembly.

To manipulate Otx2 and Gbx2 function in a temporally controlled manner, we used hormone-inducible constructs where their homeodomain is fused to the engrailed repressor domain (EnR; Glavic, 2002). These constructs have previously been shown to mimic the effects of full length Otx2 and Gbx2 mRNAs in mid/hindbrain organiser positioning (Glavic et al., 2002) and in the subdivision of the pre-placodal region (Steventon et al., 2012). In addition, the Gbx2-EnR-GR construct rescues knock-down phenotypes of Gbx2 morpholinos (Li et al., 2009). Activation with dexamethasone (DEX) leads to the translocation of constitutive repressor forms into the nucleus thus causing repression of all or a subset of Otx2/Gbx2 target genes. To target the otic region, Gbx2-EnR-GR was injected into the A3 blastomere at the 32-cell stage. In the absence of DEX, expression of the otic marker Pax2 at stage 26 is normal (Fig. 2I). In contrast, upon addition of DEX at stage 18, Pax2 continues to be expressed, but the otic vesicle is of abnormal morphology (Fig. 2J). We next assessed cell behaviour using the same grafting strategy described above. Gbx2-EnR-GR mRNA was co-injected with nuclear RFP and the labelled posterior PPR was...
placodal presence of DEX (Fig. 3I, +DEX=35). Thus, during placode condensation, activation of Otx2 targets is required for persistent cell movements and for cells to integrate into the forming lens.

**Gbx2 targets are required for directed movement of cells into the otic placode**

Do similar directional movements occur as the posterior placode territory divides into discrete placodes? nRFP-labelled posterior PPRs were grafted into unlabelled host embryos of the same stage to follow placode cell movements in the posterior PPR. Cell tracking reveals that otic or epibranchial progenitors (Fig. 4A,B; green, Movie 4) display an increase in both track length (Fig. 4A) and overall displacement (Fig. 4B) when compared epidermal cells (Fig. 4A,B; red, Movie 4). Although there is no significant difference in the average speed (Fig. 4F) of
Fig. 4. Gbx2 targets are required for directional cell movements into the otic placode. (A,B) Automatic tracks of PPR cells sorted into placodal (green) or non-placodal (red) cells based on final position and overlaid onto the final frame of the movie (A). (B) Cell displacements, arrows indicate the displacement of cells from their initial to final positions. (C) An example of five cells that were automatically tracked in a region posterior and ventral to the forming otic placode. (D,E) Summary diagrams of all tracked cells. To compare movements of cells that were automatically tracked in a region posterior and ventral to the forming otic placode. (D,E) Summary diagrams of all tracked cells. To compare the directionality of cells, tracks were translated as in Fig. 3. (F,G) Student t-tests (2-tailed) of otic, epibranchial (epi) and non-placodal cell movements to analyse mean velocity (F; \( \mu_l \), with the exception of nuclear RFP, which was used at 200 pg/μl. The authors confirm that all experiments within this article conform to the relevant regulatory standards of the UK.

Materials and Methods

Embryo techniques

Xenopus embryos were obtained as described previously (Steventon et al., 2012) and staged according to Nieuwkoop and Faber (1967). Plasmids were linearized; RNA transcribed using SP6 or T7 RNA polymerases, and the GTP cap analogue (Harland and Weintraub, 1985). To repress Gbx2 and Otx2 downstream targets, their homeodomain was fused to the repressor domain of engrailed and the hormone-inducible GR domain (Otx2-EnR-GR and Gbx2-EnR-GR; Glavic et al., 2002). All mRNAs were mixed with diethylpyrocarbonate (DEPC)-treated water to a concentration of 500 pg/μl, with the exception of nuclear RFP, which was used at 200 pg/μl. All embryos were prepared, hybridized and stained as previously described (Steventon et al., 2012) and staged according to Nieuwkoop and Faber (1967). Plasmids were linearized; RNA transcribed using SP6 or T7 RNA polymerases, and the GTP cap analogue (Harland and Weintraub, 1985).

In situ hybridisation

Xenopus embryos were prepared, hybridized and stained as previously described (Harland, 1991), and NBT/BCIP or BCIP alone were used to reveal the signal. The gene was expressed in the correct location, however, cells are more wide spread and placodes have abnormal shapes suggesting that Gbx2 and Otx2 control cell behaviour. Whether the endogenous proteins act as transcriptional repressors or activators is currently unknown and will require the identification of their targets in the future.

Cell movements have previously been shown to accompany placode formation in different species (Bhat and Riley, 2011; Kwan et al., 2011; Streit, 2002; Theveneau et al., 2013). A previous study in Xenopus showed that limited directional movements are observed within the pre-placodal region at mid-to-late neurula stages (Pieper et al., 2011). Here we show that at later stages in Xenopus directional movements do indeed accompany the formation of morphologically distinct placodes. We demonstrate for the first time that unlike future epidermal cells placode progenitors move directionally and that this behaviour is important for the assembly of placodes with normal morphology.

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Competing interests

The authors declare no competing or financial interests.
Author contributions
Conceptualization: B.S., R.M. and A.S.; Methodology: B.S., R.M. and A.S.;
Investigation: B.S.; Writing - original draft preparation: B.S.; Writing - review and editing: B.S., R.M. and A.S.; Visualization: R.M. and A.S.; Supervision: R.M. and A.S.; Funding acquisition: R.M. and A.S.

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Supplementary information
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