Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

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

The b-HLH transcription factor Hes3 participates in neural plate border formation by interfering with Wnt/β-catenin signaling

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ARTICLE INFO

Keywords:
Melanocytes
Neural crest
Progenitors
Hes3
Sox10
Xenopus

ABSTRACT

Hes3 belongs to the Hes basic helix-loop-helix family of transcriptional repressors that play central roles in maintaining progenitor cells and regulating binary cell fate decisions in the embryo. During *Xenopus laevis* development, *hes3* is expressed in the embryonic ectoderm in a horseshoe shape domain at the edge of the developing neural pate. Hes3 mis-expression at early neurula stage blocks neural crest (*snai2*, *sox8*, *sox9* and *sox10*) and cranial placode (*six1* and *dmrta1*) gene expression, and promotes neural plate (*sox2* and *sox3*) fate. At tailbud stage, these embryos exhibited a massive up-regulation of both *sox8* and *sox10* expression, associated with an increase in genes important for melanocytes differentiation (*mitf* and *dct*). Using a hormone inducible construct we show that Hes3 does not induce a pigment cell differentiation program *de novo*, rather it maintains progenitor cells in an undifferentiated state, and as Hes3 expression subsides overtime these cells adopt a pigment cell fate. We demonstrate that mechanistically Hes3 mediates its activity through inhibition of Wnt/β-catenin signaling, a molecular pathway critical for neural crest specification and pigment cell lineage differentiation. We propose that Hes3 at the edge of the neural plate spatially restricts the response to mesoderm-derived Wnt ligands, thereby contributing to the establishment of sharp boundaries of gene expression at the neural plate border.

1. Introduction

At the end of gastrulation the neural plate border (NPB) defines a competence domain, established between the neural plate (NP), prospective central nervous system, and non-neural ectoderm, future epidermis. Within this domain signaling events progressively direct the emergence of two major embryonic structures, the neural crest (NC) and the pre-placodal region (PPR). In the trunk, the NC gives rise to neurons of the peripheral nervous system and pigment cells of the skin, while in the head region the NC forms cartilages and bones of the face and contributes to cranial ganglia (reviewed in Huang and Saint-Jeannet, 2004; Bronner and LeDouarin, 2012). The PPR segregates into cranial placodes that form the paired sensory organs (olfactory epithelium, inner ear and lens), the adenohypophysis, and a subset of cranial ganglia that provide sensory innervation to the orofacial complex (reviewed in Baker and Bronner-Fraser, 2001; Streit, 2007; Park and Saint-Jeannet, 2010; Schlosser, 2010). NPB formation is regulated by several signaling molecules of the Wnt, fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families that need to be precisely modulated in space and time to generate the NC and the PPR (reviewed in Stuhlmiller and García-Castro, 2012; Bae and Saint-Jeannet, 2014; Saint-Jeannet and Moody, 2014; Singh and Groves, 2016). These signaling molecules in turn differentially activate the expression of a subset of transcription factors that uniquely define the molecular identity of these two cell populations and their derivatives (reviewed in Grocott et al., 2012; Simões-Costa and Bronner, 2015).

Hes genes are vertebrate homologs of *Drosophila hairy* and *enhancer of split* genes, forming a family of seven members (*Hes 1–7*). They encode basic helix-loop-helix transcriptional repressors that play essential roles in controlling the maintenance and expansion of stem cell populations, and the timing of their differentiation (reviewed in Kobayashi and Kageyama, 2014). For example, Hes1 and Hes5 expression inhibits neuronal differentiation and promotes proliferation of neural progenitors in the mouse embryonic brain (Ohtsuka et al., 2001), and inactivation of *hes1*, *hes3* and *hes5* genes accelerates neuronal differentiation, depleting prematurely the pool of neural progenitors (Hatakeyama et al., 2004).

In *Xenopus*, Hairy2 also known as Hes4 is expressed at the NPB and has been proposed to mediate Notch signaling during NC induction (Glavic et al., 2004). Hairy2 knockdown blocked NC induction,

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https://doi.org/10.1016/j.ydbio.2018.07.011
Received 15 May 2018; Received in revised form 2 July 2018; Accepted 13 July 2018

proliferation and differentiation (Nagatomo and Hashimoto, 2007). Hairy2 gain-of-function had a very similar phenotype at early stage, however later in development these cells activated a glial cell differentiation program, suggesting that sustained Hairy2 expression maintained these progenitors in an undifferentiated state (Nichane et al., 2008). During *Xenopus* cranial placode development, another Hes family member, Hes8, has been shown to mediate the inhibition of proneural and neuronal differentiation gene expression in response to Notch signaling (Riddiford and Schlosser, 2017).

In this manuscript we report the expression and function of Hes3 during *Xenopus* development. *hes3* is expressed at the edge of the NP, and Hes3 mis-expression blocks NC gene expression and promotes neural fate. Interestingly later in development these embryos show ectopic activation of a pigment cell differentiation program, suggesting that Hes3 maintains NC progenitors in an undifferentiated state, and overtime these progenitors acquire a pigment cell fate as Hes3 expression subside. Mechanistically we provide evidence that Hes3 mediates its activity by modulating the response to Wnt/β-catenin signaling.

2. Materials and methods

2.1. Plasmid constructs

Xenopus laevis Hes3.L (accession number: XM_018226116.1) full coding region was generated by polymerase chain reaction (PCR) and fused in frame to the human glucocorticoid receptor (GR) ligand binding domain to generate an pCS2+*Hes3GR* construct using the following primers, forward: 5′-ATCGATGCCACCATGGGGACACA TTCCGAACCACGAGAAGA-3′ and reverse: 5′-CTCGAGCCATGGTCT CCACACGTCTTGG-3′. The activity of the fusion protein can regulated by addition of dexamethasone to the culture medium of whole embryos or animal explants (Kolm and Sive, 1995).

2.2. Xenopus embryo injections, explants culture, and dexamethasone treatment

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (1967) and raised in 0.1X NAM (Normal Amphibian Medium; Slack and Forman, 1980). This study was performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The procedures were approved by New York University Institutional Animal Care and Use Committee, under animal protocol # 150201. *hes3GR*, *nog* (Smith and Harland, 1992), *wnt8* (Christian et al., 1991), *ctnnb1* (Funayama et al., 1995) and *β-galactosidase* (*β-gal*) mRNAs were synthesized *in vitro* using the Message Machine kit (Ambion, Austin TX). In whole embryo experiments, synthetic *hes3GR* mRNA (500 pg) was injected in one blastomere at the 2-cell stage (NF stage 2). Injected embryos were cultured in 0.1 X normal amphibian medium (NAM; Slack and Forman, 1980) containing 10 μM dexamethasone (Dex; Sigma-Aldrich, St. Louis, MO) from NF stage 10.5 or 17. Siblings injected with *hes3GR* and cultured in the absence of Dex were used as control. For *wnt8* and *ctnnb1*, plasmid DNA was injected to prevent axis duplication (100 pg and 200 pg, respectively). To identify the injected side, 500 pg of *β-gal* mRNA was coinjected as a lineage tracer and embryos were analyzed by *in situ* hybridization at the appropriate stage. Morpholino antisense oligonucleotides (MO) to knockdown Hes3 function were purchased from GeneTools (Philomath, OR). We used two translation blocking MOs targeting Hes3.L (HES3LMO: AGGTTCGGAATGTGTCCCCATGTTT and HES3MO2: TCCCCATGTTTGAAGGAGTTGGTTT), one splice blocking MO targeting the intron 1-exon 1 junction of Hes3.L and Hes3.S (HES3SMO1: CGCGCAGTACAATATACTGACCTTT), and one translation blocking MO targeting Hes3. S (HES3MO3: CTTGAGGTTCAGAG TGAGTCCCCAT). The MOs were injected in one blastomere at the 2-cell stage either separately or in combination (HES3MO1 +Hes3MO3), to target both Hes3.L and Hes3.S. For the axis duplication assay, embryos were injected with 4 pg of *wnt8* mRNA in the equatorial region in both ventral blastomeres at 4-cell stage (NF stage 3) and analyzed at NF stage 32. For animal explant experiments, both blastomeres at the two-cell stage were injected in the animal pole region, with various combinations of *nog* (400 pg), *wnt8* (50 pg) and *hes3GR* (1 ng). Then explants were dissected at the late blastula stage and immediately cultured *in vitro* for several hours in NAM 0.5X plus 10 μM Dex. For whole embryo injections and animal cap explant assays each experiment was performed on at least three independent batches of embryos.

2.3. Lineage tracing and whole-mount in situ hybridization

Embryos at the appropriate stage were fixed in MEMFA and stained for Red-Gal (Research Organics; Cleveland, OH) to visualize the lineage tracer (*β-gal* mRNA) on the injected side and processed for *in situ* hybridization. Antisense digoxygenin-labeled probes (Genius kit; Roche, Indianapolis IN) were synthesized using template cDNA encoding *hes3*, *snai2* (Mayor et al., 1995), *sox8* (O'Donnell et al., 2006), *sox9* (Spokony et al., 2002), *sox10* (Aoki et al., 2003), *foxd3* (Sasai et al., 2001), *pax3* (Bang et al., 1997), *zic1* (Mizuseki et al., 1998), *dmrta1* (Huang et al., 2005), *sox2* (Mizuseki et al., 1998), *sox3* (Penzel et al., 1997) and *dct* (Aoki et al., 2003). Whole-mount *in situ* hybridization was performed as described (Harland, 1991; Saint-Jeannet, 2017).

2.4. qRT-PCR analysis

For each sample, total RNAs were extracted from 10 animal cap explants using the RNeasy micro RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. During the extraction procedure the samples were treated with DNase I, to eliminate possible contamination by genomic DNA. The amount of RNA isolated was quantified by measuring the optical density using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). qRT-PCR was performed with 10 ng of total RNAs from animal caps using *Power* SYBR® Green RT-PCR Master Mix (Applied Biosystems, Foster City, CA) on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following primer sets: *snai2*, *sox2, six1,* pax3 (Hong et al., 2007), *sox10* (F: CTGTGAACACAGCATGCAAA; R: TGGCCAACTGACCATGTAAA), *drmta1* (F: TGGAATGTTAC GGGATCCAT; R: AGGCCACTGTGGGACTATTG), *mitf* (F: CA AGAGATGCTGCAAAACCA; R: GCTGTTGGGGAGTCAGACAT) and *dct* (F: AACGGGAAGGAATGAGTGTG; R: GGTCACCAGCCGAT TGTAGT). The PCR conditions were as follows: denaturation 95 °C (15 s), annealing and extension at 60 °C (1 min) for 40 cycles.

3. Results

3.1. Hes3 belongs to the Hes family of transcriptional repressors

Xenopus laevis Hes3 possesses an open reading frame encoding 204 amino acids (Fig. 1A). Outside the b-HLH domain and the N-terminal groucho domain at the amino acid level Hes3 proteins have limited conservation across species (Fig. 1A). Overall *Xenopus laevis* Hes3 (XP_018081605.1) shares 35% identity with human HES3 (NP_001019769.1; Katoh and Katoh, 2004), 32% identity with mouse Hes3 (NP_032263.2; Sasai et al., 1992), 35% identity with zebrafish Hes3 (NP_571155.1; Hans et al., 2004) and 82% identity with *Xenopus tropicalis* Hes3 (XP_004916246.1). Phylogenetically *Xenopus laevis* Hes3 falls into the same clade as mouse and human HES3 (Fig. 1B).

3.2. Hes3 is expressed at the edge of the developing neural plate

We analyzed the expression of *hes3* by whole mount *in situ* hybridization (ISH) using digoxygenin-labeled RNA probes. At early