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Xaml1/Runx1 is required for the specification of Rohon-Beard sensory neurons in Xenopus

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ABSTRACT

Lower vertebrates develop a unique set of primary sensory neurons located in the dorsal spinal cord. These cells, known as Rohon-Beard (RB) sensory neurons, innervate the skin and mediate the response to touch during larval stages. Here we report the expression and function of the transcription factor *Xaml1/Runx1* during RB sensory neurons formation. In Xenopus embryos *Runx1* is specifically expressed in RB progenitors at the end of gastrulation. *Runx1* expression is positively regulated by Fgf and canonical Wnt signaling and negatively regulated by Notch signaling, the same set of factors that control the development of other neural plate border cell types, i.e. the neural crest and cranial placodes. Embryos lacking Runx1 function fail to differentiate RB sensory neurons and lose the mechanosensory response to touch. At early stages Runx1 knockdown results in a RB progenitor-specific loss of expression of *Pak3*, a p21-activated kinase that promotes cell cycle withdrawal, and of *N-tub*, a neuronal-specific tubulin. Interestingly, the pro-neural gene *Ngnr1*, an upstream regulator of *Pak3* and *N-tub*, is either unaffected or expanded in these embryos, suggesting the existence of two distinct regulatory pathways controlling sensory neuron formation in Xenopus. Consistent with this possibility *Ngnr1* is not sufficient to activate *Runx1* expression in the ectoderm. We propose that *Runx1* function is critically required for the generation of RB sensory neurons, an activity reminiscent of that of *Runx1* in the development of the mammalian dorsal root ganglion nociceptive sensory neurons.

Introduction

The ectoderm of the vertebrate embryos can be divided into three regions at the end of gastrulation: the neural plate, which is the precursor of the central nervous system, the non-neural ectoderm forming the epidermis, and the neural plate border (NPB) that arises at the boundary between the neural plate and the non-neural ectoderm. The NPB is the source of two important cell populations: the neural crest (NC) and the pre-placodal ectoderm (PE). The NC is located lateral to the neural plate but is excluded from its most anterior region. NC cells will migrate in the periphery and give rise to a broad array of derivatives including craniofacial structures, the pigment cell lineage and peripheral nervous system (Le Douarin et al., 2004). The PE is restricted to the anterior-most region of the neural plate and lateral to the NC. The PE will eventually segregate into individual cranial placodes to give rise to the sensory organs in the head (Park and Saint-Jeannet, 2010a; Schlosser, 2010).

In anamniotes such as the frog *Xenopus laevis* the NPB gives rise to two additional cell populations: the hatching gland (HG) cells and a group of primary neurons known as Rohon-Beard (RB) sensory neurons. The HG is located in the outer layer of the ectoderm of the anterior neural folds, medial to the prospective NC. The HG produces proteolytic enzymes, which digest the vitelline envelope and jelly coat to release the tadpole into the environment (Drysdale and Elinson, 1991). The RB sensory neurons arise from the posterior-most region of the NPB. At the end of neurulation, these neurons are located in the dorsal spinal cord and innervate the skin to mediate the escape response to touch at the larval stages (Roberts and Smyth, 1974). Later in development RB neurons will undergo apoptosis (Lamborghini, 1987) and their function will be assumed by the NC-derived dorsal root ganglia neurons (reviewed in Roberts, 2000). Genes typically expressed in RB sensory neuron progenitors are also detected in two additional primary neuron subpopulations confined to a more medial region of the neural plate, the primary interneurons

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and motoneurons. This is the case, for example, of the basic helix loop helix (b-HLH) gene neurogenin-related-1 (*Ngnr1*; Ma et al., 1996), and the neural-specific tubulin gene, *N-Tubulin* (*N-Tub*; Chitnis et al., 1995). The absence of molecular markers restricted to RB sensory neuron progenitors has made it difficult to analyze the more unique requirements of this population of primary neurons in terms of specification and differentiation.

The gene *Runx1* encodes a runt domain transcription factor with a critical role in hematopoietic stem cell formation and definitive hematopoiesis in mammals (reviewed in Swiers et al., 2010). *Runx1* is also expressed in a subpopulation of dorsal root ganglia (DRG) sensory neurons involved in pain transduction and regulates aspects of the differentiation of this group of nociceptive neurons (reviewed in Stifani and Ma, 2009). In Xenopus in addition to its expression and function in blood progenitors, *Xaml1*/*Runx1* is expressed in RB neuron precursors at the end of gastrulation (Tracey et al., 1998), and therefore represents a unique tool to analyze the development of this population of primary sensory neurons. Moreover little is known of the role *Xaml1*/*Runx1* in the formation of these mechanosensory neurons.

Here we describe the detailed expression pattern of *Runx1* in RB progenitors as compared to other primary neuron-specific genes. We characterize the regulatory inputs controlling *Runx1* expression at the NPB and analyze the consequences of Runx1 knockdown on the sensory function of Xenopus tadpoles. We also analyze the position of *Runx1* in the regulatory cascade leading to RB sensory neurons specification. Our findings indicate that *Runx1* function is critically required in RB progenitors to promote cell cycle exit and neuronal differentiation, and that *Runx1* is acting in parallel with *Ngnr1* to regulate sensory neuron formation.

Materials and methods

Plasmid constructs

Vertebrate *Runx1* genes are expressed from two alternative promoters, a distal (P1) and a proximal (P2), that encode isoforms with distinct amino-terminal sequences (Supplementary Fig. S1), here referred to as *Runx1* (accession # BC057739.1) and *Xaml1* (accession # AF035446), respectively. *X. laevis Runx1* (pCMV-Sport6) was obtained from Open Biosystems. *Xaml1* was amplified by PCR from stage 30 cDNA using primers based on the published sequence (Tracey et al., 1998), and subcloned into pGEM-T Easy (Promega). Both ORFs including 9 bp (*Runx1*) and 14 bp (*Xaml1*) upstream of the ATG were amplified by PCR and subcloned into pCS2 + expression plasmid digested with ClaI and XbaI. These two constructs were used to test the specificity of the translation blocking morpholino antisense oligonucleotides (Supplementary Fig. S1). We generated a hormone-inducible version of *Ngnr1* (Ma et al., 1996) by sub-cloning the coding region of Ngnr1 into pCS2 + GR (Ngnr1-GR). All constructs were sequenced and the corresponding proteins monitored using an in vitro transcription/translation assay.

In vitro transcription/translation

The in vitro transcription/translation coupled rabbit reticulocyte lysate system (SP6-TNT, Promega) was performed following the manufacturer recommendations (Promega), in the presence of ^{35}S-methionine. The reaction was resolved on a NuPAGE BIS-Tris gel (Invitrogen). The gel was dried using GelAir Drying System (Bio-Rad) and the product of the TNT reaction was detected by exposure onto a BioMax film (Kodak).

Morpholino antisense oligonucleotides

β-Catenin (β-catMO; 25 ng; Heasman et al., 2000), Wnt8 (Wnt8MO; 30 ng; Park and Saint-Jeannet, 2008), Fgf8a (Fgf8aMO; 50 ng; Fletcher et al., 2006), Pax3 (Pax3MO; 50 ng; Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005), Zic1 (Zic1MO; 45 ng; Hong and Saint-Jeannet, 2007; Sato et al., 2005) and control (CoMO; 60 ng) morpholino antisense oligonucleotides were purchased from Gene-Tools LLC (Philomath, OR). To interfere with Runx1 function we used two translation blocking and a splice blocking morpholinos. Runx1MO (CACTATGTGAGGCCATTGCGTTTCC) and Aml1MO (GGGATACGCATCACAACAAGCCTGG) specifically block translation of *Runx1* (P1 promoter) and *Xaml1* (P2 promoter) mRNA, respectively. The specificity of Runx1MO and Aml1MO was tested in an in vitro transcription/translation coupled rabbit reticulocyte lysate assay (Supplementary Fig. S1). Based on *Xenopus tropicalis* genome information (Ensembl Gene ID: ENSXETG00000014140), several intronic regions within *Runx1* were selected as candidate sites for a splice-inhibitory morpholino. Primers flanking these introns in the *X. laevis Xaml1*/*Runx1* mRNA sequence were used to amplify *X. laevis* genomic DNA fragments, which were cloned into pGEM-T (Promega) and sequenced. We designed a splice-inhibitory morpholino (Runx1SMO: AAACAGAGCCAGGGTCTTACCTTGA) targeting the Exon1–Intron1 junction (Supplementary Fig. S1).

Embryo injections and in situ hybridization

Embryos were staged according to Nieuwkoop and Faber (1967). Fgf8a (2 pg; Christen and Slack, 1997), Notch-ICD (0.5 ng; Chitnis and Kintner, 1996) and Ngnr1-GR (0.5 ng; Perron et al., 1999) mRNAs were synthesized in vitro using the Message Machine kit (Ambion, Austin, TX). Synthetic mRNA and morpholino antisense oligonucleotides were injected in the animal pole of 2-cell stage embryos. All embryos were co-injected with the lineage tracer β-gal mRNA (β-gal; 0.5 ng) to identify the injected side. *Ngnr1-GR* (0.5 ng) injected embryos were treated with 10 μM dexamethasone (Sigma) in NAM 0.1X at stage 10.5 or stage 12.5, as described (Gammill and Sive, 1997). Untreated sibling embryos were used as a control (not shown). For in situ hybridization embryos were fixed in MEMFA and were successively processed for Red-Gal (Research Organics) staining to detect β-gal activity, and in situ hybridization. Antisense DIG-labeled probes (Genius kit, Roche) were synthesized using template cDNA encoding *Xaml1*/*Runx1* (Tracey et al., 1998), *Xhe* (Katagiri et al., 1997), *N-Tub* (Chitnis et al., 1995), *Pak3* (Souopgui et al., 2002), *Ngnr1* (Ma et al., 1996), *Islet1* (Brade et al., 2007), *Krox20* (Bradley et al., 1993), *Snail2* (Mayor et al., 1995), *XK81* (Jonas et al., 1989), *Kv1.1* (Burger and Ribera, 1996) and *Ccndx* (Chen et al., 2007). Whole-mount in situ hybridization was performed as previously described (Harland, 1991). For in situ hybridization on sections, embryos were fixed in MEMFA for 1 h, embedded in Paraplast+, 12 μm sections cut on a rotary microtome and hybridized with the appropriate probes as described (Henry et al. 1996). Sections were then briefly counter stained with Eosin.

Proliferation assay

For phosphohistone H3 detection (Saka and Smith, 2001), Sox9MO-injected albinos embryos were fixed in MEMFA. Embryos were incubated successively in α-phosphohistone H3 antibody (Upstate Biotechnology; 1 μg/ml) and anti-rabbit IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch; 1:1000). Alkaline phosphatase activity was revealed using NBT/BCIP (Roche). Fluorescein lysine dextran (FLDX; MW 10,000, Molecular Probes) was used as a lineage tracer to identify the injected side.

Touch response assay

During embryogenesis *Xenopus* embryos develop a dual touch sensory system largely mediated by RB sensory neurons (Roberts and Smyth, 1974). The touch response assay and quantification was