Anosmin-1 is essential for neural crest and cranial placodes formation in Xenopus

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A B S T R A C T

During embryogenesis vertebrates develop a complex craniofacial skeleton associated with sensory organs. These structures are primarily derived from two embryonic cell populations the neural crest and cranial placodes, respectively. Neural crest cells and cranial placodes are specified through the integrated action of several families of signaling molecules, and the subsequent activation of a complex network of transcription factors. Here we describe the expression and function of Anosmin-1 (Anos1), an extracellular matrix protein, during neural crest and cranial placodes development in Xenopus laevis. Anos1 was identified as a target of Pax3 and Zic1, two transcription factors necessary and sufficient to generate neural crest and cranial placodes. Anos1 is expressed in cranial neural crest progenitors at early neurula stage and in cranial placode derivatives later in development. We show that Anos1 function is required for neural crest and sensory organs development in Xenopus, consistent with the defects observed in Kallmann syndrome patients carrying a mutation in ANOS1. These findings indicate that anos1 has a conserved function in the development of craniofacial structures, and indicate that anos1-depleted Xenopus embryos represent a useful model to analyze the pathogenesis of Kallmann syndrome.

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1. Introduction

The vertebrate head is characterized by a complex craniofacial skeleton and paired sensory organs. These structures are derived from two embryonic cell populations, neural crest and cranial placodes, that are specified at the anterior border of the neural plate at the end of gastrulation. There is abundant literature indicating that molecules of the fibroblast growth factor (Fgf), Wnt and bone morphogenetic protein (Bmp) families must be precisely modulated to specify neural crest and cranial placodes in the embryo [1–4]. These growth factors in turn activate a unique repertoire of transcription factors that are responsible for initiating the differentiation program of each one of these cell populations [5,6]. Among these transcription factors, Pax3 and Zic1 are especially critical to promote neural crest and cranial placode fates [7–12]. To characterize the molecular events downstream of Pax3 and Zic1, several years ago we performed a microarray screen using Xenopus animal cap explants expressing varying combinations of these factors [13]. Here we describe the expression and function of one of these targets Anosmin-1. Anosmin-1 is a secreted molecule of the extracellular matrix, encoded by the anos1 gene. In human, mutations in ANOS1 cause Kallmann syndrome, a condition characterized by craniofacial defects, anosmia, deafness and hypogonadotropic hypogonadism [14,15]. Using knockdown approaches in Xenopus embryos and animal cap explants we show that Anosmin-1 is required for neural crest specification and for cranial placode-derived sensory organs formation, consistent with defects observed in Kallmann syndrome patients. There is no anos1 ortholog in mouse therefore anos1-depleted Xenopus embryos may represent a unique model to analyze the etiology and pathogenesis of Kallmann syndrome.

2. Materials and methods

2.1. Plasmid constructs and morpholino antisense oligonucleotides

The ORF of Xenopus laevis anos1 was amplified by PCR from neurula stage embryo cDNA using the following primers, F: 5′-
ATGTTGCTAGGGAGCCAGGC-3' and R: 5'-TCAGTACTCTCTGGG-GATGG-3'. Anos1 (anos1MO; 5'-GAGAACTCCTGCTCCACGAGGATCAT-3'), Pax3 (pax3MO; [7]), and Zic1 (zic1MO; [8]) morpholino anti-sense oligonucleotides (MOs) were purchased from GeneTools (Philomath, OR). The pax3GR and zic1GR hormone-inducible constructs were generated as previously described [9]. Synthetic mRNAs encoding pax3GR, zic1GR, noggin, wnt8a, anos1, and β-galactosidase [9,16] were synthesized in vitro using the Message Machine kit (Ambion, Grand Island NY).

2.2. Embryos, injections and explants culture

*Xenopus laevis* embryos were staged as previously described [17] and raised in 0.1X NAM (Normal Amphibian Medium; [18]). The procedures were approved by NYU Institutional Animal Care and Use Committee, under animal protocol # 150201. Embryos were injected in one blastomere at the 2-cell stage with MOs and synthetic mRNAs together with 500 pg of β-galactosidase mRNA in 100 pg, Snai2 [20], Sox10 [21], Sox2 [19], Six1 [23], Foxi4.1 [24], Dmrta1 [25], Pax8 [26], Emx2 [27], Ebf2 [28] and Foxe1 [29].

2.3. Lineage tracing, in situ hybridization and histology

*Xenopus* embryos were fixed in MEMFA and processed for Red-Gal (Research Organics, Cleveland OH) staining to visualize β-galactosidase prior to ISH. Whole-mount ISH was performed as previously described [19]. Digoxigenin (DIG)- labeled antisense RNA probes (Roche Diagnostics, Indianapolis, IN) were synthesized using template cDNA encoding Anos1, Snai2 [20], Sox10 [21], Sox2 [22], Six1 [23], Foxi4.1 [24], Dmrt1 [25], Pax8 [26], Emx2 [27], Ebf2 [28] and Foxe1 [29].

2.4. Western blot analysis

For Western blots, embryos were injected at the 2-cell stage with mRNA (500 pg) encoding a myc tagged version of anos1 along with increasing amounts of anos1MO and cultured up to stage 15. Pools of 10 embryos were homogenized in lysis buffer (0.5% Triton with increasing amounts of anos1MO and cultured up to stage 15. 2.4. Western blot analysis

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2.5. RNA preparation and qRT-PCR

Total RNAs from embryos were extracted using the RNasy Micro Kit (Qiagen, Valencia CA). For mRNA extraction from animal cap explants, pools of 8 explants were homogenized and mRNAs were isolated using Dynabeads® mRNA DIRECT™ Micro Kit (Invitrogen). cDNA synthesis from total RNA and mRNA were performed using Superscript VILO cDNA Synthesis Kit (Invitrogen, Grand Island, NY). qRT-PCR was performed on an Eco Real-Time PCR System (Illumina, San Diego CA) using the primers shown in Table 1, and the Power SYBR Green PCR Master Mix (Invitrogen). The reaction mixture consisted of 10 μl of Power SYBR Green PCR Master Mix, 200 nM primers, and 2 μl of cDNA in a total volume of 10 μl. The PCR conditions were as follows: 95 °C for 10min; 40 cycles at 95 °C for 10sec and at 60 °C for 30sec. The ΔΔCT method was used to analyze the qRT-PCR results. Each reaction included a standard curve of serial dilution points (in 10-fold increments) of test cDNA. odc1 was used for normalization.

3. Results

3.1. Anos1 is expressed in the neural crest and cranial placodes

Anosmin-1 (Anos1, formerly Kal1; [30]), an extracellular matrix protein, was independently isolated in two microarray screens designed to identify targets of Pax3 and Zic1 [13,31]. Both transcription factors are necessary and sufficient to specify the neural crest and placodes [7–9]. By ISH anos1 transcripts are first detected at the early neurula stage (NF stage 14) at the lateral edge of the anterior neural plate, in the prospective cranial neural crest region (Fig. 1A). As the neural plate closes, anos1 remains spatially confined to the neural crest territory (Fig. 1B and C). As development proceed, anos1 is expressed in the pharyngeal arches, somites, and anteriorly at the midline in the prospective pituitary gland (Fig. 1D–F). At the tailbud stage (NF stage 27) anos1 transcripts persist in the pharyngeal arches, somites, and anterior pituitary, and appeared to accumulate in the ventral aspect of the otic vesicles (Fig. 1G and H). Using qRT-PCR, we compared the temporal expression profile of anos1 to that of snai2, a well-established early neural crest gene [22]. We found that anos1 is maternally expressed (NF stage 8), and towards the end of gastrulation (NF stage 12) the escalation of snai2 and anos1 expression follows a very similar pattern (Fig. 1I).

3.2. Anos1 is a true target of Pax3 and Zic1

To confirm that anos1 is a target of Pax3 and Zic1, we performed perturbation experiments in the embryo using pax3MO and zic1MO [7–9]. Pax3 or Zic1 knockdown resulted in a dramatic reduction or loss of anos1 expression in a vast majority of the embryos.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>anos1</td>
<td>5'- GGGAGTGTTTGTAGTGATGAGG -3'</td>
<td>5'- GGGATGGTGGTTTAGTGATGAGG -3'</td>
</tr>
<tr>
<td>snai2</td>
<td>5'- CACCATGTCATTCTGTCGAGG -3'</td>
<td>5'- AGGCACTGAAAGGTAGAGA -3'</td>
</tr>
<tr>
<td>sox2</td>
<td>5'- TACCCCTCTTCCTCAGCCATC -3'</td>
<td>5'- CGACATGTGCAGTCTGCTTT -3'</td>
</tr>
<tr>
<td>sox8</td>
<td>5'- ACGCTCCTGAGGTCTTCTG -3'</td>
<td>5'- GGGATGGTTTGTAGTGATGAGG -3'</td>
</tr>
<tr>
<td>odc1</td>
<td>5'- ACATTTTACCTTTTTCGAAC -3'</td>
<td>5'- TGGTCCCAAGGCTAAAGTTG -3'</td>
</tr>
<tr>
<td>ef1α</td>
<td>5'- ACCCTCCTCTCTTTTTCAC -3'</td>
<td>5'- CTTAGTACTCTCTGGG-GATGG -3'</td>
</tr>
</tbody>
</table>
Fig. 1. Developmental expression of anos1 by whole-mount ISH. (A–C) At the neurula stage (NF stage 14–17), anos1 is detected in the prospective neural crest territory (white arrowheads). (D–F) At stage 23, anos1 is now more broadly expressed, to include the somites (green arrowheads), otic vesicle (red arrowhead), the anterior pituitary (yellow arrowhead) in addition to the branchial arches (white arrowheads). (G–H) Later in development (NF stage 27) anos1 persists in all these tissues. (A–C) dorsal views, anterior to top. (D, G) lateral views, dorsal to top, anterior to left. (E, F, H) frontal views, dorsal to top. The embryonic stages (NF) are indicated in the lower right corner of each panel. (I) Relative expression levels of anos1 and snai2 analyzed by qRT-PCR at the indicated stages. The values were normalized to odc1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
demonstrating that both factors are independently required for anos1 expression (Fig. 2A). In animal cap explants (Fig. 2B), expression of pax3GR and/or zic1GR showed that Pax3 and Zic1 are sufficient to induce anos1 expression and their activity is additive, while Pax3 and Zic1 synergistically activate snai2 (Fig. 2C). Taken together, these results indicate that anos1 is expressed in Xenopus cranial neural crest and its expression is regulated by Pax3 and Zic1.

3.3. Anos1 is necessary but not sufficient for neural crest development

To determine whether anos1 function is implicated in neural crest development we performed anos1 knockdown using MOs. anos1MO was designed to specifically interfere with translation of anos1 mRNA. The activity of the MO was confirmed by Western blot. In embryos injected with mRNA encoding a myc-tagged version of Xenopus anos1, increasing doses of anos1MO blocks Anos1 protein accumulation (Fig. 3A). Unilateral injection of anos1MO at the 2-cell stage led to a reduction of expression of several neural crest genes including snai2 and sox10 at stage 14 (Fig. 3B and C). The loss of neural crest genes correlated with an expansion of sox2 expression domain in morphant embryos (Fig. 3B). We also tested the function of Anos1 in an animal cap explant assay. Activation of the Wnt/β-catenin pathway in conjunction with attenuation of BMP signaling induces neural crest genes in animal cap explants [32]. We found that the induction of the neural crest genes, snai2 and sox8, by wnt8a and noggin was significantly repressed in Anos1-depleted (MO-injected) animal cap explants (Fig. 3D). The reduction in snai2 and sox8 expression was associated with an increase in sox2 expression, consistent with a loss of neural crest fate. Therefore, in the embryos and in animal cap explants Anos1 function is required to specify the neural crest.

We also performed gain-of-function experiments. Unilateral injection of anos1 mRNA at the 2-cell stage did not significantly affect snai2 and sox10 expression levels, although their expression appeared shifted laterally (Fig. 3B and C). Moreover, in animal cap explant anos1 expression was unable to activate snai2 or sox8 expression (not shown). These results support the view that anos1 participates in neural crest formation downstream of Pax3 and Zic1, but is not sufficient to activate a neural crest development program.

**Fig. 2. anos1 is a target of Pax3 and Zic1.** (A) Injection of pax3MO (40 ng) or zic1MO (40 ng) reduces anos1 expression at the neurula stage. Dorsal views, anterior to top. The injected side is to the right as indicated by the presence of the lineage tracer (Red-Gal). The graph indicates the percentage of embryos with normal (white) or reduced/lost (red) anos1 expression. The number of embryos analyzed is indicated on top of each bar. (B) mRNA encoding pax3GR and zic1GR (100 pg each), alone or in combination were injected into both blastomeres in the animal pole at the 2-cell stage. At the blastula stage (stage 9), animal cap (AC) explants were dissected and cultured for 8 h in the presence of dexamethasone (+dex). (C) anos1 and snai2 expression in pax3GR and zic1GR injected AC explants analyzed by qRT-PCR.
3.4. Anos1 is essential for sensory placodes development

In human, mutations in ANOS1 have been associated with sensory deficits, including anosmia and hearing loss, and hypogonadotrophic hypogonadism due to malfunction of the gonadotropin releasing hormone (GnRH) system [15]. To determine whether Anosmin-1 is also critical for sensory organs development in Xenopus we performed unilateral injection of anos1MO at the 2-cell stage. These injections resulted in the reduction/loss of expression of snail2 and sox10, and a lateral expansion of sox2 expression domain. Injection of Xenopus anos1 mRNAs (3 ng) did not significantly affect snail2 and sox10 expression levels, although their expression domain was shifted laterally. The expression of sox2 was only marginally expanded in these embryos. Dorsal views, anterior to top. The injected side is to the right (Red-Gal). (C) The graphs indicate the percentage of embryos with normal (white), reduced/lost (red) expression. The number of embryos analyzed is indicated on top of each bar. (D) In explants, the induction of snail2 and sox8 by co-injection of noggin (200 pg) and wnt8a (10 pg) mRNA is dramatically reduced in the context of embryos injected with anos1MO (40 ng). This reduction in neural crest genes expression is associated by an increase in sox2 expression. The values were normalized to ef1a. A representative experiments is shown from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Here we describe the expression and function of Anosmin-1 (Anos1) during early Xenopus laevis development. Anos1 was identified as a target of Pax3 and Zic1 [13,31], two important regulators of neural crest and cranial placode fates in the embryo [7–12]. anos1 is first detected at the early neurula stage at the lateral edges of the anterior neural plate, in the cranial neural crest forming region. Later in development, anos1 expression persists in neural crest cells as they populate the branchial arches, and is activated in the prospective nasal and otic placodes, anterior
pituitary, and in the somites. MO-mediated knockdown of Anosmin-1 resulted in a reduction of neural crest and cranial placode genes expression suggesting that Anosmin-1 is required for the specification of both cell populations.

ANOS1 encodes a 100-kDa glycoprotein of the extracellular matrix with great affinity for cell membrane heparin sulfate proteoglycans. It is highly conserved across species, with anos1 orthologs present in Drosophila, C. elegans, zebrafish, and most mammals. Interestingly, anos1 has not been identified in rat and mouse [30]. Anos1 is composed of several functional domains including a cysteine rich region, a whey acidic protein-like domain (typically present in serine protease inhibitors), four consecutive domains Fibronectin-like type III domains (implicated in cell-cell adhesion) and an histidine-rich C-terminal region [30,33]. Anos1 has been implicated in cell-cell adhesion, cell migration and differentiation of multiple cell types in the nervous system. Anos1 regulates migration of immortalized GnRH producing neurons as well as neuronal and oligodendrocyte precursors. It has also a role in axon guidance, neurite outgrowth and the genesis of axon collaterals from neurons in the olfactory system [34–36].

Molecules of the Bmp, Fgf and Wnt families have been implicated in neural crest and cranial placode induction [1–4], however little is known how the activity of these growth factors is controlled in the extracellular space. Functional studies in vivo and in vitro have shown that Anos1 physically interacts with fibroblast growth factor receptor 1 (Fgfr1) and its ligand Fgf2 to activate signaling [37–40]. More recently, the role of Anos1 was evaluated during cranial neural crest formation in chicken embryos. This study showed that Anos1 upregulates Fgf8 and Bmp5 gene expression, and binds directly to Fgf8, Bmp5 and Wnt3a to modulate their activity locally and promote cranial neural crest formation [41]. While anos1 is also expressed in the anterior neural fold region in chicken embryos, it is unclear whether its activity is also involved in the regulation of cranial placode development. Our observations indicate that in addition to the cranial neural crest, Anos1 plays a critical role in cranial placodes formation, as Anos1 knockdown affects the development of adenohyphyseal, olfactory and otic placode derivatives. While we did not detect anos1 transcripts in the placodal region at the early neurula stage, Anos1 knockdown resulted in the reduction of two pan-placodal genes, six1 and foxi4.1. Since Anos1 is a secreted protein it is likely that knocking down its function in the cranial neural crest will also affect adjacent cell populations, including the formation of cranial placode progenitors, which depends on similar signaling events for their specification.

ANOS1 is the gene responsible for X-linked Kallmann’s syndrome [42,43]. This condition is the result of defects in the development of GnRH and olfactory neurons, both cell types originating from the nasal placode. As a consequence these patients suffer from hypogonadotropic hypogonadism and anosmia. Associated defects include renal agenesis, cleft lip with or without cleft palate, hearing loss and abnormal tooth development. The genetics and cellular pathogenesis of Kallmann syndrome are still incompletely understood [15,44], therefore it is essential to develop suitable experimental model systems to gain insights into the molecular basis underlying this condition. We propose that anos1-depleted Xenopus embryos may represent an excellent model to investigate the pathogenesis of Kallmann syndrome and the mechanisms of action of Anos1.

Conflicts of interest

The authors declare no conflict of interest.

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