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Stable knock-down of vomeronasal receptor genes in transgenic *Xenopus* tadpoles

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Abstract

Xenopus V2R (xV2R), a family of G-protein-coupled receptors with seven transmembrane domains, is expressed in the *Xenopus* vomeronasal organ (VNO). There are six subgroups of xV2R, one of which, xV2RE, is predominantly expressed in the VNO. To understand the function of xV2R during VNO development, we developed a new method to achieve stable siRNA-suppression of the V2RE genes by introducing siRNA expression transgenes into the genomes of unfertilized eggs. We found that some of the derived transgenic tadpoles lacked VNOs and that their olfactory epithelium was fused. With the exception of one tadpole, expression of xV2RE was not detected in morphologically abnormal mutant tadpoles, although the olfactory marker protein and the olfactory receptors were expressed. These results suggest that we successfully produced transgenic tadpoles in which xV2RE expression was stably suppressed by siRNA, and that xV2RE plays a role in the morphogenesis of olfactory organs.

Keywords: siRNA; V2R; VNO; Olfactory epithelium; *Xenopus laevis*; Transgenic

Most vertebrates possess two anatomically distinct olfactory organs: the olfactory organ and the vomeronasal organ (VNO) [1]. The epithelium of the olfactory organ is responsible for the conventional sense of smell whereas the VNO is generally believed to detect pheromones that provide information about the social and reproductive status of other individuals and that elicit innate behavior and neuroendocrine responses [2–4].

In rodent VNOs, V1R and V2R, two large and divergent families of G-protein-coupled receptors with seven transmembrane domains, are expressed in subsets of the vomeronasal receptor cells [5–8]. A member of the V1R family responds to a mouse pheromone [9], suggesting that this V1R is a pheromone receptor. Although the function of V2R has not yet been elucidated, it is thought that this receptor family also mediates pheromone responses [10].

Recent gene-targeting approaches show that axons from vomeronasal receptor cells expressing the same receptor coalesce into 10–30 glomeruli [11], and that the capacity to converge these glomeruli depends on the ability to express the chosen vomeronasal receptor [12]. These observations suggest that the vomeronasal receptors function as

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axon guidance molecules. The vomeronasal receptors are also proposed to play a role in the chemosensory guidance of sperm [13]. Consequently, current evidence indicates that the vomeronasal receptors are multifunctional proteins.

Phylogenetically, VNOs first appeared in amphibians [14]. Therefore, analysis of the vomeronasal receptors of *Xenopus laevis* (xV2R) might provide information on the original or new functions of vomeronasal receptors. However, use of a gene knock-out strategy to analyze vomeronasal receptors, whether applied to single or several xV2R genes, has limited effectiveness because each xV2R gene is expressed only in a subset of the vomeronasal cells. Moreover, the pseudotetraploid genome of *Xenopus laevis* makes it quite difficult to generate gene knock-out animals. Furthermore, loss-of-function experiments involving the introduction of antisense RNAs [15] or siRNAs [16] have been of limited value because their expression is transient.

Previously, we identified six subgroups in the xV2R family of *Xenopus laevis* [17]. We found that one subgroup of xV2R (xV2RE) was predominantly expressed in VNOs and that there are conserved sequences within the xV2RE genes. Thus, stable knock-down of xV2R genes might be achieved if transgenes that stably express siRNAs targeted against these conserved sequences can be integrated into the *Xenopus* genome. Stable knock-down would consequently suppress the expression of these genes in a large number of the vomeronasal receptor cells during the course of VNO development. We generated transgenic tadpoles by introducing siRNA expression plasmids into the genomes of unfertilized *Xenopus* eggs. Our observations of the olfactory organs in transgenic tadpoles suggest that the xV2R genes play a role in the morphogenesis of the olfactory organs.

Materials and methods

Animal maintenance. Xenopus laevis tadpoles and adults were maintained in the laboratory at 20 °C. Tadpoles were raised on SERA Micron (Sera Heinsberg, Germany), and frogs on salmon pellets (Oriental Kobo, Tokyo) and crickets (*Gryllus bimaculatus*). Tadpoles were staged according to Nieuwkoop and Faber [18].

Construction of plasmids. To construct the siRNA expression plasmids, two 19-nucleotide sequences were used as target sequences: SeqA, 5′-TCACCTTCTCCATAGCTGT-3′; SeqB, 5′-CTGGACCTTCACACTTACC-3′. A third sequence, SeqA′ (5′-CCAGCTTGTCCTTAGCTCT-3′), was derived from SeqA with five base mismatches (underlined) and used as a negative control. The siRNA expression cassettes expressed sense and antisense target sequences as a single unit. A 17-nucleotide loop sequence (TAGAATTACATCAAGGGAGAT) was inserted between the sense and antisense nucleotides as described previously [19]. T_6 was added to the 3′-terminus for transcription termination. These cassettes were inserted into the *Apa*I–*Eco*RI site of the pSilencer 1.0-U6 vector (Ambion, Austin, TX). The siRNA plasmids constructed in this way were designated pA, pB, and pA′, respectively. The CMV-EGFP transcription unit, with a polyadenylation signal at the 3′-end of the coding sequence, was amplified by PCR and inserted into the *Not*I sites of pA, pB, and pA′ in the reverse transcription direction with respect to the U6 promoter. The original *Not*I site at the 3′-end of the coding sequence of EGFP in pEGFP-N (Clonetech, Palo Alto, CA) was removed prior to performing PCR. The plasmids thus constructed were designated pA/E, pB/E, and pA′/E, respectively (Fig. 1C). They were linearized with *Sca*I and used for DNA transformation of sperm nuclei.

For constructing the xV2R1 expression plasmid, the coding sequences of the initial methionine and the next 38 residues of bovine rhodopsin were amplified from the bovine retina lambda cDNA library (Stratagene, Palo Alto, CA) by PCR and were joined to a full-length xV2R1 cDNA [17]. The rhodopsin-tag of xV2R1 is required to translocate xV2R1 across the plasma membrane for functional expression [20] and was used to detect xV2R1 expression with an anti-rhodopsin antibody. The chimeric rhodopsin-xV2R1 gene was introduced into a pCAGGS expression plasmid under a strong CAG promoter [21].

DNA transfection and immunohistochemistry. HEK 293T cells were grown at 37 °C in DMEM supplemented with 10% fetal bovine serum. Then, 0.5 μg of rhodopsine-xV2R1 plasmid, with or without 0.5 μg siRNA plasmid, was transfected into semiconfluent HEK293T cells. Two days after transfection, cells were fixed with 2% paraformaldehyde/PBS and

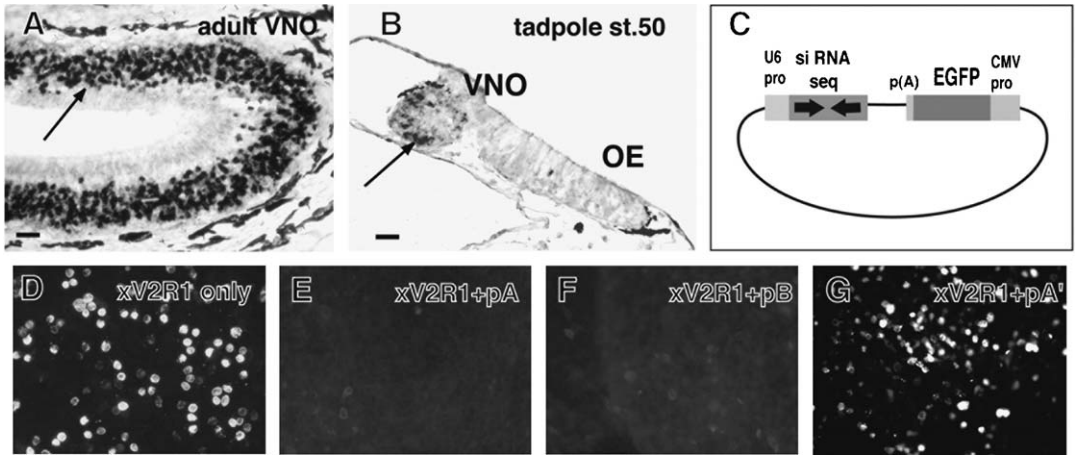

Fig. 1. Expression of xV2RE in *Xenopus* VNOs and the silencing effect of siRNA-expressing plasmids on the expression of xV2R1 in 293T cells. Cross-sections of adult (A) and tadpole VNOs at stage 50 (B) were hybridized with digoxigenin-labeled V2RE-specific antisense probe as described previously [17]. A schematic illustration of the siRNA-expressing plasmid (C). Arrows in (A,B) indicate the sites of xV2R1 expression. Chimeric rhodopsin-xV2R1 plasmid was transfected into HEK 293T cells with pA (E), pB (F), pA′ (G) or without siRNA plasmid (D), and expression of xV2R1 was detected by anti-rhodopsin antibody as described in the Materials and methods. Black spots on the periphery of the OE and VNO in cross-sections (A,B) are melanocyte aggregates. Bars indicate 50 μm.

subjected to immunostaining as described elsewhere [22]. Expression of chimeric rhodopsin-xV2R1 was detected using a polyclonal anti- (bovine) rhodopsin rabbit antibody (SLS), at a 1:500 dilution, followed by Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes), at a 1:200 dilution.

Generation of transgenic tadpoles. Transgenic tadpoles were generated using the method of Kroll and Amaya [23]. Briefly, the dorsal lymph sac of adult female frogs was injected with 700 U human chorionic gonadotropin and unfertilized eggs were collected. Linearized pA/E, pB/E, and pA′/E were mixed with sperm nuclei in vitro in order to introduce plasmid DNAs into the sperm nuclei. Transformed nuclei were transplanted into unfertilized eggs, which were then incubated, and the embryos were raised to stage 50–51.

EGFP detection. EGFP expression in tadpoles was monitored using a fluorescent dissecting microscope (MZ FLIII, Leica, Atlanta, GA) equipped with a digital camera (DP70, Olympus, Tokyo).

In situ hybridization and staining with hematoxylin/eosin. The preparation of sections of the adult vomeronasal organ has been described elsewhere [17]. Tadpoles were fixed overnight in 4% paraformaldehyde/PBS and embedded using routine procedures in OCT compound or in paraffin. In situ hybridization procedures were performed as described previously [17]. OCT compound-embedded sections (6 μm) were treated with 4% paraformaldehyde/PBS for 5 min, permeabilized with 0.2% TritonX/PBS for 10 min, and treated with 3 μg/ml of proteinase K for 15 min at 37 °C. Sections were re-fixed with 4% paraformaldehyde/PBS for 5 min, and 0.2% glycine/PBS for 5 min, treated with 0.2 N HCl for 20 min, and neutralized with 0.1 M Tris–HCl (pH 8.0) for 1 min. Treated samples were hybridized with digoxigenin-labeled cRNA probes at 55 °C overnight and washed with 5× SSC/50% formamide at 55 °C for 30 min. After treatment with 2 μg/ml RNase for 30 min, samples were washed twice with 2× SSC and twice with 0.2× SSC at 55 °C. After treatment with blocking reagent (Roche, Basel), slides were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche). Probe-positive signals were detected and visualized by chromogenic development in NBT (4-nitro blue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (Roche).

The cDNA fragments coding for the genes of olfactory receptors type 1 (xOR1) [24] and olfactory marker protein (xOMP-2) [25] were isolated from the *Xenopus* olfactory and vomeronasal cDNA library [17] by PCR using the following primers: xOMP sense primer, CTG AAACCTCAGAGATGGAG; xOMP antisense primer, TGGGTCAG CGCCATCTAAG; xOR1 sense primer, ATGACCATCTCAATGT CTAATC; xOR1 antisense primer, CGGTTGGGTCCACAGTAAGG. The amplified fragments were cloned into pBSSK vector (Toyobo). The cDNA clone (E-1), which was used for the detection of the expression of xV2RE genes, was described previously [17]. The antisense and sense RNA probes were synthesized using T7 and T3 RNA polymerases, and were labeled with digoxigenin as described previously [17]. Paraffin-embedded samples were sectioned (15 μm) and stained with hematoxylin/eosin.

Results

To date, six subgroups (subgroup A to F) of the V2R gene family have been identified in *Xenopus laevis*. Of these, genes of the xV2R subgroup E (xV2RE) are expressed in a large proportion of VNO neurons (Fig. 1A, arrow) [17]. These genes are also clearly expressed in VNOs of *Xenopus* tadpoles at stage 50 (Fig. 1B, arrow). To suppress xV2RE expression, we constructed two U6 promoter-driven hairpin-type siRNA expression plasmids, pA and pB. These

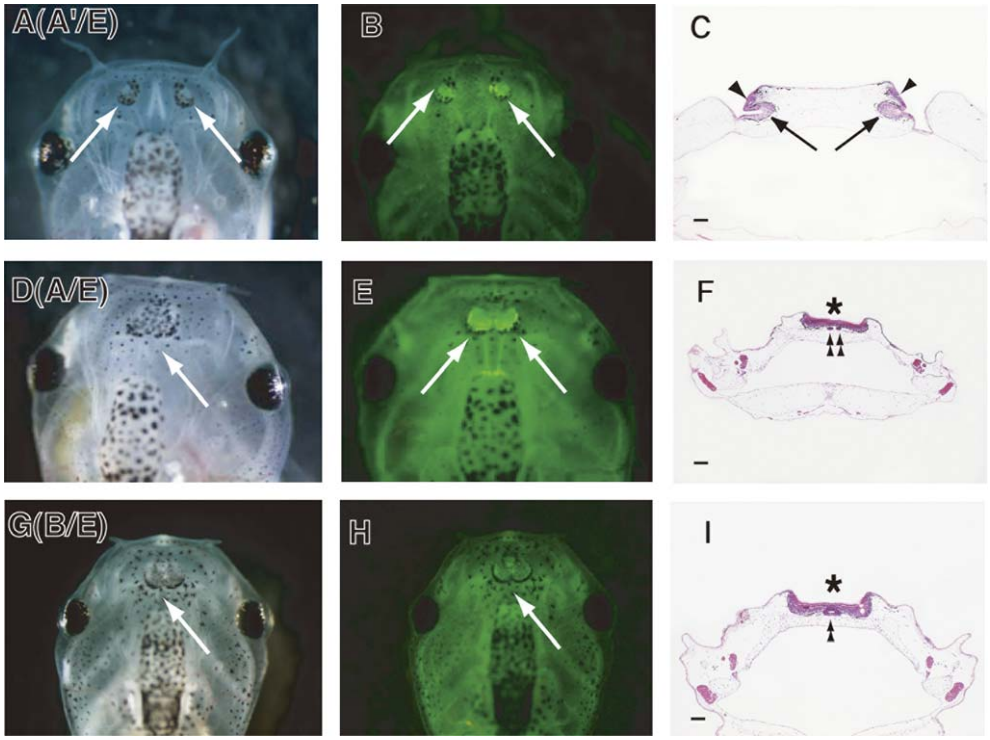

Fig. 2. Morphological features of olfactory organs of transgenic tadpoles. Photographs of pA′/E-derived EGFP-positive tadpole with morphologically normal nostrils (A) and pA/E- (D) and pB/E (G) -derived EGFP-positive tadpoles with fused nostrils at stage 50–51 (white arrows indicate nostrils). EGFP expression in tadpoles of A (B), D (E), and G (H) was monitored using a fluorescent dissecting microscope (white arrows indicate OE). Paraffin-embedded sections (15 μm) of pA′/E-derived EGFP-positive tadpoles with normal nostrils (C) and pA/E- (F), pB/E (I) -derived EGFP-positive tadpoles with fused nostrils were stained with hematoxylin/eosin. The VNO (arrows), OE (arrowheads), fused OE (asterisks), and Jacobson's glands (double arrowhead) are shown. Bars indicate 50 μm.

respectively target two different sequences, SeqA and SeqB (see Materials and methods), in the region corresponding to the coding sequence of the transmembrane domain of xV2R1, a member of the xV2RE gene family [17]. Both of the target sequences were conserved within the xV2RE genes (data not shown). pA′, having a five-base mismatch in SeqA of pA, was constructed as a negative control (see Materials and methods).

The silencing effect of these plasmids was analyzed by transfecting a chimeric rhodopsin-xV2R1plasmid, with or without siRNA expression plasmids, into cultured HEK293Tcells and expression of xV2R1 was detected using anti-rhodopsin antibody as described in the Materials and methods. The expression of xV2R1 (Fig. 1D) was efficiently suppressed by cotransfection with pA (Fig. 1E) and pB (Fig. 1F), but not with pA′ (Fig. 1G).

Next, we constructed transgenic tadpoles containing these siRNA-expressing transgenes together with an EGFP gene as a marker to facilitate detection of transgenic tadpoles. Plasmids A/E, pB/E, and pA′/E were introduced into the genomes of unfertilized eggs and transgenic tadpoles containing siRNA-expressing transgenes were selected by EGFP fluorescence (see Materials and methods). We obtained 51 pA/E-, 126 pB/E-, and 71 pA'/E-derived EGFP-positive tadpoles at stage 50–51, and found that some of the pA/E- (4 out of 51) and pB/E-derived (5 out of 126) EGFP-positive tadpoles exhibited fused nostrils (Fig. 2D and G, arrows), although no such abnormalities were seen in any of the pA′/E-derived EGFP-positive controls (0 out of 71) (Fig. 2A, arrows). EGFP expression in the olfactory epithelium (OE) shows that the pair of OEs seemed to be fused in all abnormal pA/E and pB/E mutants (Figs. 2E and H, arrows). The analysis of the sections of these mutants indicates that the morphological characteristics of fused nostrils in pA/E and pB/E mutants were similar: the VNOs were absent and the OEs were

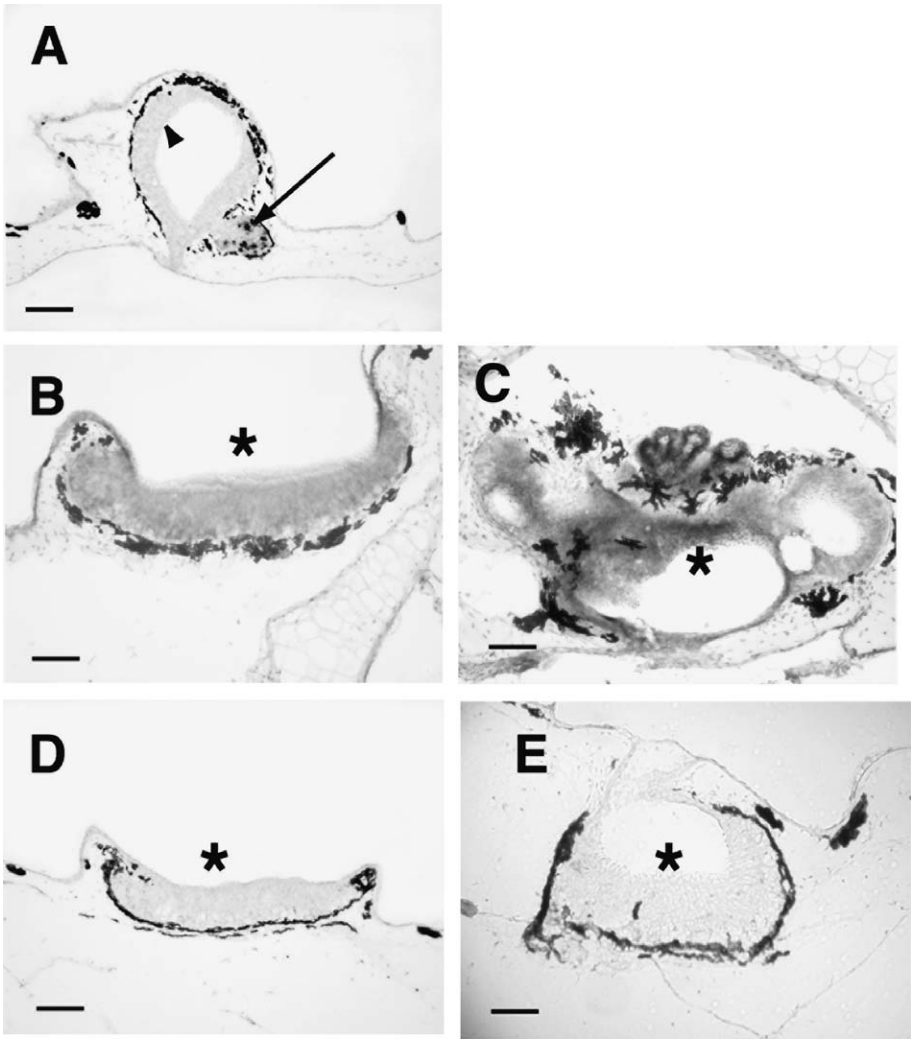

Fig. 3. Expression of the xV2RE genes in the olfactory organs. Cross-sections of pA′/E-derived EGFP-positive tadpoles with normal nostrils (A) and pA/E- (B,C), pB/E (D,E) -derived EGFP-positive tadpoles with fused nostrils. The sections were hybridized with digoxigenin-labeled V2RE-specific antisense probe as described in the Materials and methods. The VNO (black arrow), OE (arrowhead), and fused OE (asterisks) are shown. Black spots on the periphery of the OE and VNO in cross-sections (A–E) are melanocyte aggregates. Bars indicate 100 μm.

fused (Fig. 2F and I, asterisks, and unpublished observations). In situ hybridization analysis of olfactory organs (see Materials and methods) confirmed that xV2RE was not expressed in 2 of 3 pA/E nor in both pB/E mutants (Fig. 3B–E), although distinct expression of xV2RE was observed in VNOs of all EGFP-positive tadpoles that had normal nostrils (10 pA/E, 8 pB/E, and 12 pA′/E) (Fig. 3A, arrow). Thus, expression of xV2RE was suppressed in 4 of the 5 mutant tadpoles. These results therefore strongly suggest that the abnormality was caused by siRNA-mediated inhibition of xV2RE expression.

The drastic effects of siRNA-mediated suppression raise the possibility that the differentiation of the olfactory neurons might be inhibited by these siRNAs. We therefore analyzed the expression patterns of OR1 and olfactory marker protein (OMP), which are regarded as good markers for mature olfactory neurons. *Xenopus* OR1 (xOR1) is generally thought to detect water-soluble odorants and is specifically expressed in the mature olfactory neurons situated in the middle cavity (MC) of adult *Xenopus laevis* [24] (Fig. 4A–C, arrow). xOR1 expression is present in the normal tadpole OE at stage 50–51 (Fig. 4D, arrows). In situ hybridization analysis showed that expression of xOR1 could be detected in both pA/E and pB/E mutant tadpoles that displayed fused nostrils (Fig. 4E and F, arrows). Next, we analyzed the expression of *Xenopus* OMP-2 (xOMP-2) by in situ hybridization. Normally, xOMP-2 expression is specifically detected in the mature olfactory neurons of the principal cavity (PC) and MC but not in VNO of *Xenopus* adults [25] (Fig. 4A–C, arrows). It is also expressed in the OE but not in the VNO of normal tadpoles (Fig. 4D, arrow). As shown in Fig. 5D, xOMP-2 was clearly expressed in the OE of both pA/E and pB/E mutant tadpoles with fused nostrils (Fig. 5E and F, arrows). Thus, although the morphology of the OE was aberrant, the expression of xOR1 and

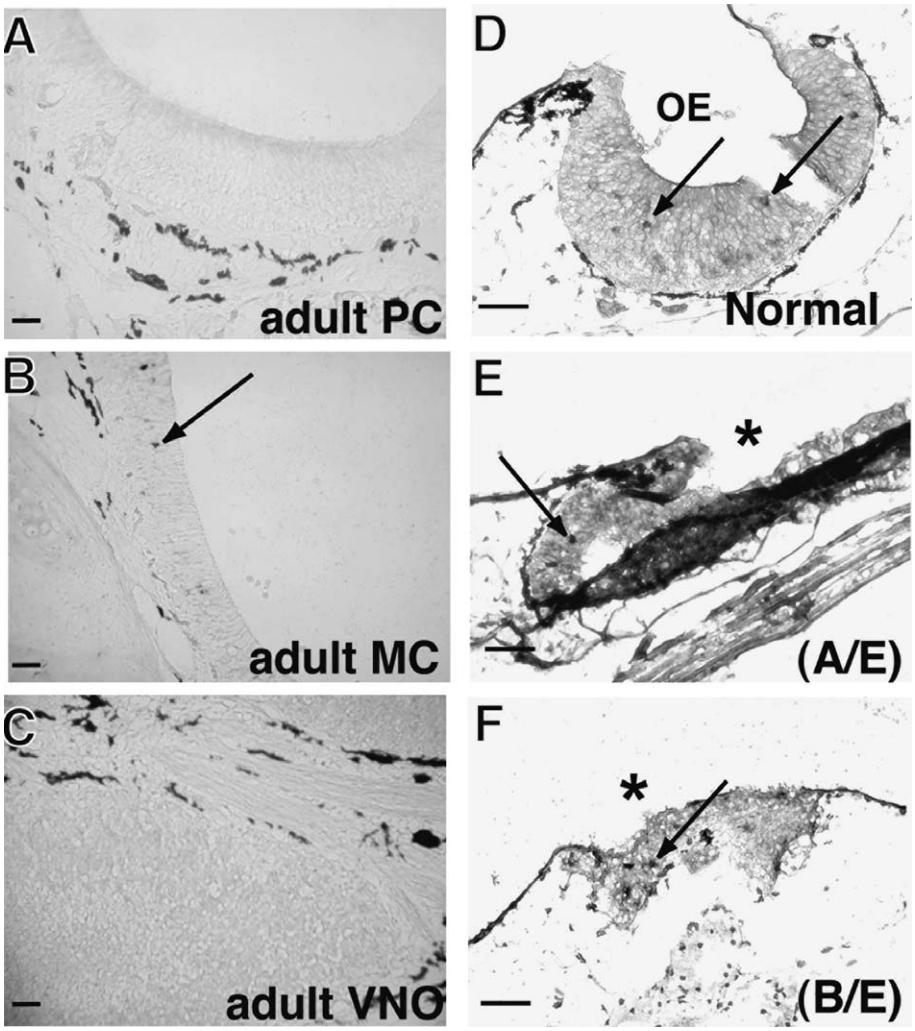

Fig. 4. Expression of xOR1 in the olfactory organs. Cross-sections of normal adult principal cavity (PC) (A), middle cavity (MC) (B), VNO (C), the normal OE in the tadpole at stage 50–51 (D), pA/E- (E), and pB/E (F) -derived EGFP-positive tadpoles with fused nostrils. The sections were hybridized with digoxigenin-labeled xOR1-specific antisense probe as described in the Materials and methods. The fused OE (asterisks) and the expression sites of xOR1 (arrows) are shown. Bars indicate 50 μm.

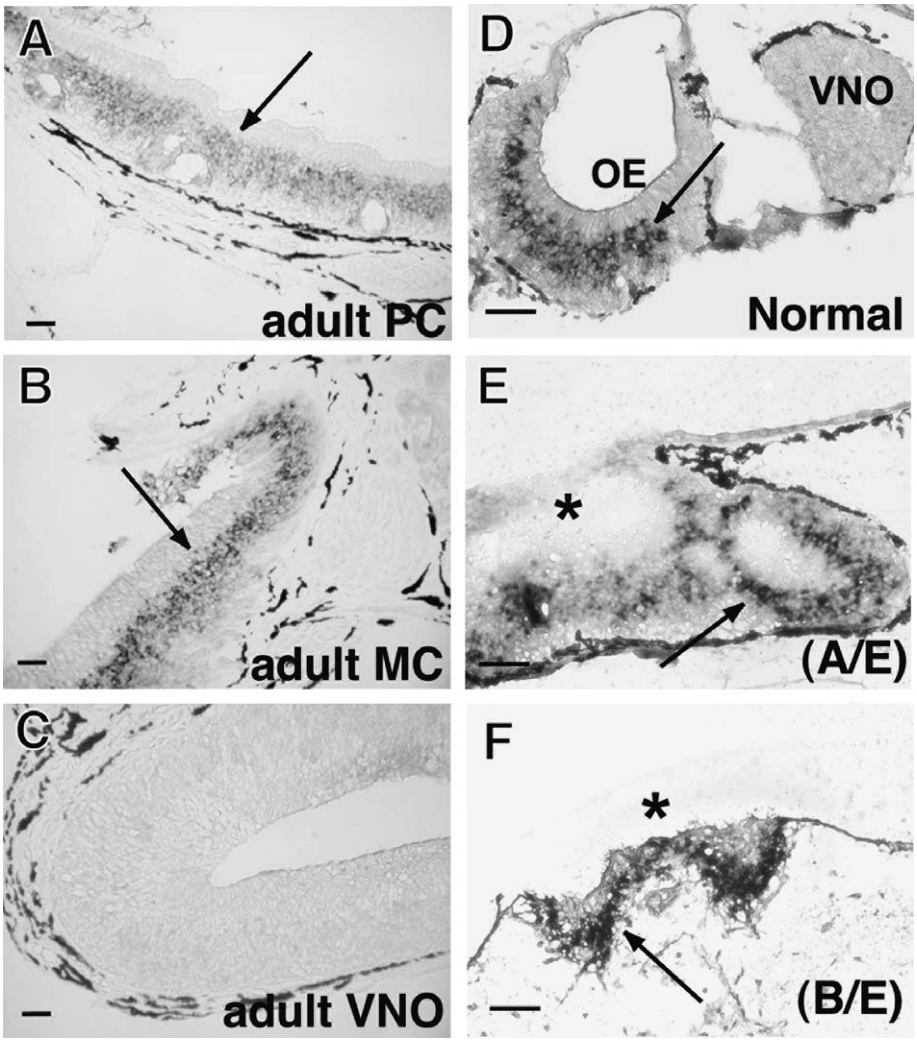

Fig. 5. Expression of xOMP-2 in the olfactory organs. Cross-sections of normal adults PC (A), MC (B), VNO (C), the normal olfactory organs in the tadpole at stage 50–51 (D), pA/E- (E), and pB/E (F) -derived EGFP-positive tadpoles with fused nostrils. The sections were hybridized with digoxigenin-labeled xOMP-2-specific antisense probe as described in the Materials and methods. The fused OE (asterisks) and the expression sites of xOMP-2 (arrows) are shown. Bars indicate 50 μm.

xOMP-2 indicated that mature olfactory neurons seemed to be developing in the mutant tadpoles.

These results suggest that we successfully generated transgenic tadpoles in which the expression of xV2RE was stably suppressed by siRNA and that, with the exception of one tadpole, malformation of the olfactory organs was associated with suppression of xV2RE expression. Given these findings, we propose that xV2RE plays a role in the development of the vomeronasal receptor cells and in the morphogenesis of olfactory organs.

Discussion

We developed a method for generating transgenic *Xenopus* tadpoles using a nuclear transplantation technique in which siRNA expression transgenes were introduced into the genomes of unfertilized eggs. The advantage of our method is that, for the first time, it becomes feasible to achieve stable suppression of gene expression during the course of the development in pseudotetraploid *Xenopus laevis*. This method may provide new insights into gene function during *Xenopus laevis* development, and will also enable the creation of lines of gene knock-down transgenic frogs.

We used two siRNAs that targeted two different regions corresponding to conserved coding sequences within the xV2RE genes and obtained transgenic tadpoles displaying similar olfactory organ mutations, i.e., absence of VNO structures and fused OEs (Fig. 2). The expression of xV2RE was suppressed in their mutated olfactory organs with the exception of one tadpole (Fig. 3), and no abnormalities were observed in negative controls (Figs. 2 and 3). These results strongly suggest that the abnormal features of the olfactory organs were caused by siRNA-mediated suppression of xV2RE expression, and that xV2RE plays a role in the develop-

ment of the vomeronasal cells and in the morphogenesis of the olfactory organs.

From the point of view of expression of genes such as xOR1 and xOMP-2, the olfactory neurons seemed to be developing in the mutant tadpoles (Figs. 4E and F, 5E and F), despite the fact that the mutant tadpoles displayed an abnormal fusion of the OEs (Fig. 2 and 3). As only a small number of cells express xV2RE in the OE at stage 50–51 [17], it is difficult to understand how suppression of xV2RE expression in these few cells can have such a drastic effect on morphology. The role of xV2RE in the morphogenesis of the olfactory organs will therefore be elucidated using a line of V2RE-gene knock-down transgenic *Xenopus*.

The expression of the other subgroups of xV2R could not be detected in the mutant tadpoles presumably because disappearance of many xV2RE-expressing cells somehow interrupted differentiation of the vomeronasal cells that expressed other subgroup of xV2R. It should be noted that even though SeqA′, with a five-base mismatch of SeqA, did not interfere with the expression of xV2RE1 in cultured cells, the expression of genes belonging the D and F subgroups might be suppressed by the siRNA because they have only four- and two-base mismatches in the corresponding sequence, respectively (data not shown). If this would be the case, the D and F subgroups may play similar roles to that of subgroup E in the development of the vomeronasal receptor cells and in the morphogenesis of the olfactory organs.

Del Punta et al. [26] reported that the VNOs of knock-out mice lacking 16 intact V1R genes did not show obvious morphological changes, implying that the V1R genes were not involved in the morphogenesis of olfactory organs. Therefore, insofar as olfactory development is concerned, the role of V2R may differ from that of V1R, although it is unclear whether a role in olfactory development is restricted to subgroup E (xV2RE) specifically, or whether all other subgroups of xV2R might also have this function.

Competing interests statement

The authors declare that they have no competing financial interests.

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