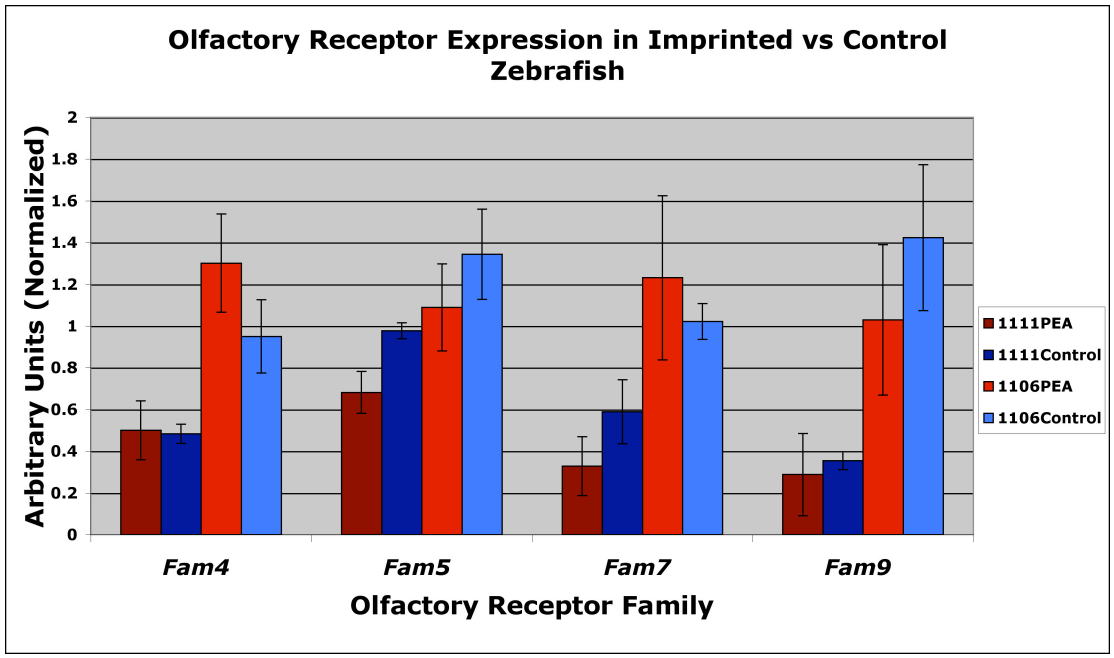
Figure A2. QRT-PCR analysis of OR expression for OR families 4, 5, 7, and 9. There was no statistical difference of OR expression between PEA (red bars) and control fish (blue bars) for any of the families tested. Similar results were observed for two different stocks (#1111 (dark shades) and #1106 (light shades)) of imprinted fish. All OR expression data was normalized to expression of β-actin .


Olfactory Receptor Expression in Imprinted vs Control Zebrafish
Arbitrary Units (Normalized)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fam4
Fam5
Fam7
Fam9
Olfactory Receptor Family
1111PEA
1111Control
1106PEA
1106Control

RNase H- Reverse Transcriptase (Invitrogen) and random primers were used for first-strand cDNA synthesis. 200ng of total RNA was added to each cDNA reaction.

Quantitative RT-PCR

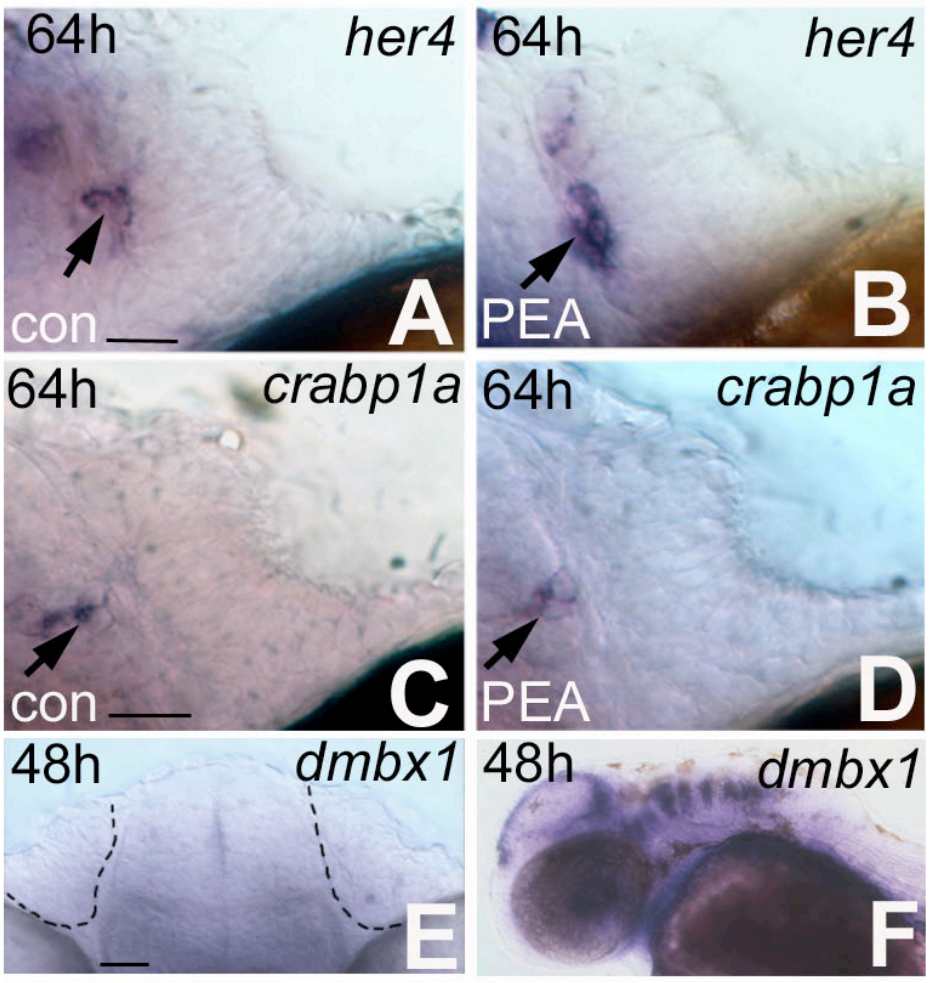
cDNA sequences of individual members from each zebrafish olfactory receptor family (families 4,5,7, and 9) were aligned with the ClustalW algorithm using MegAlign software (DNASTAR, Lasergene v6) and primers were designed to conserved regions within the coding regions of the receptor families (For primer sequences, see Table A1). Ideally, QRT-PCR primers are designed across intron-exon boundaries to avoid amplification of genomic sequence. This was impossible for the zebrafish OR genes because they do not have introns. QRT-PCR reactions were performed using a 7900HT Sequence detection system (Applied Biosystems) following the protocols in the User Bulletin #2 (updated October, 2001 by Applied Biosystems, www.appliedbiosystems.com). Results from quantitative RT-PCR were analyzed using the relative standard curve method (User Bulletin #2, Applied Biosystems, www.appliedbiosystems.com). A two-fold dilution series of cDNA made from pooled RNA isolated from untreated wild type (new wild type strain) zebrafish olfactory epithelia was used to make standard curves for all primer pairs. SYBR®-Green PCR Master Mix (Applied Biosystems) was used for detection of PCR products. Reaction mixes included 25µl SYBR®-Green Master Mix, 50nM of each primer and 15ng of cDNA in a total of 50µl. Thermocycling conditions were the same for all primer pairs: 95^{o}C for 10min; 40 cycles of the following: 95^{o}C for 30sec., 60^{o}C for 45sec. and 72^{o}C for 1min.; after cycling, additional cycles were added for dissociation curve

analysis. Dissociation curve analysis showed that only one product was present in each reaction. All reactions were run in triplicate. Reverse transcriptase minus controls were run for all samples to ensure that genomic DNA was not present in RNA samples. "No template" controls were also run for all primer pairs to ensure that there was no cDNA contamination in any of the reagents. β-actin was used as an endogenous control for all experiments. Data was analyzed using Microsoft Excel as described in the User Bulletin and OR family expression was graphed relative to β-actin expression.

MICROARRAY ANALYSIS OF CHANGES IN GENE EXPRESSION IN ADULT OLFACTORY EPITHELIA FROM PEA-IMPRINTED AND CONTROL ZEBRAFISH

The change in *otx2* expression described in chapter three was identified from expression array analysis using RNA isolated from adult OEs of PEA-imprinted and control fish. Table 3.1 presented the genes that were up-regulated 2.0-fold and higher. In addition to *otx2*, three other genes were examined for expression changes in the developing OE using *in situ* hybridization. Cellular retinoic acid binding protein 1a (*crabp1a*; 6.01-fold increase in PEA-imprinted OEs) was examined because of it was previously shown to be expressed in the developing olfactory system (Gustafson et al., 1999; Asson-Batres et al., 2003). Two other transcription factors were chosen based on known roles in the developing nervous system: diencephalon/ mesencephalon homeobox 1 (*dmbx1;* 3.9-fold increase) and hairy-related 4 (*her4;* 2.78-fold increase). These genes were cloned and digoxigenin labeled mRNA probes were made for use in *in situ* hybridization. Expression was examined in 1-3 day old PEA-exposed and control juvenile zebrafish. *her4* is

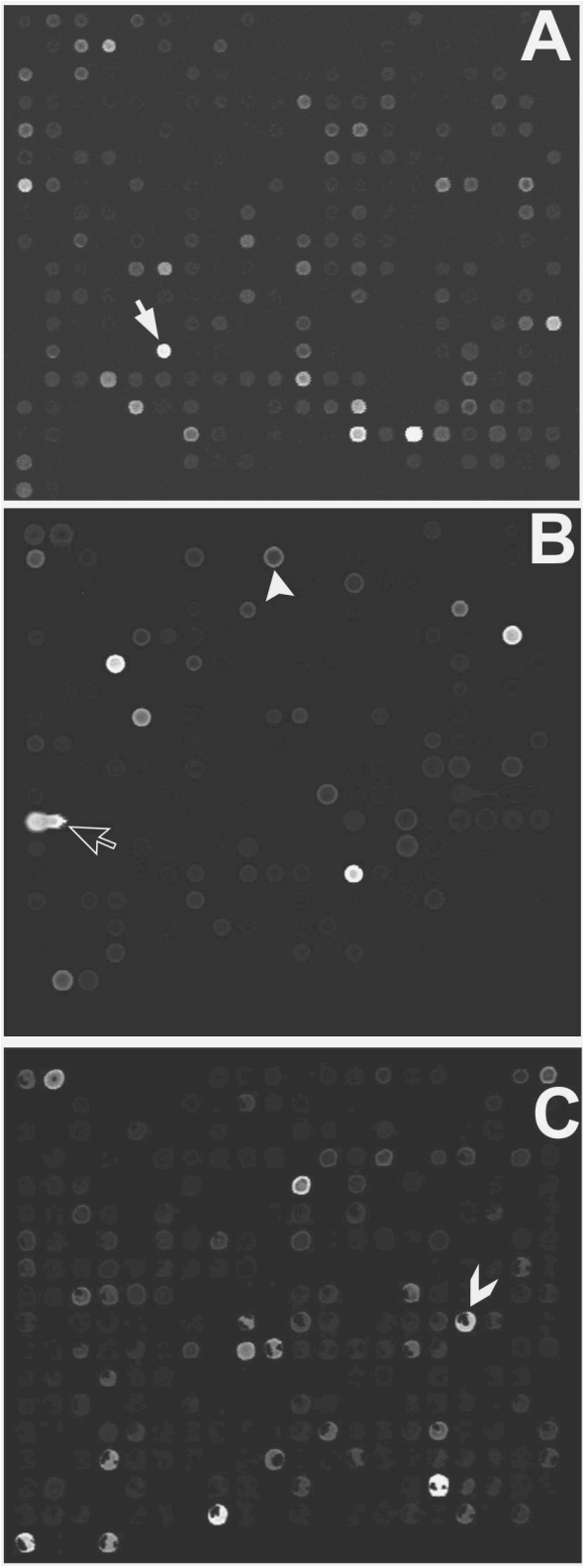
Figure A3. Expression patterns of three other genes identified using microarray analysis as being up-regulated in PEA-imprinted zebrafish. *In situ* hybridization was used to examine the expression of *her4* (A,B), *crabp1a* (C,D) and *dmbx1* (E,F) in the developing OE. Cells expressing *her4* (A,B, arrows) are found at the basal part and immediately outside of the developing OE. Fish exposed to PEA (B) show a slightly higher level of *her4* mRNA expression than controls (A). *crabp1a* (C,D, arrows) is expressed in small clusters of cells immediately outside of the developing OE. Expression is only observed starting at 56h. There was no difference in expression between PEA-exposed juveniles (D) and control siblings (C). *dmbx1* was not expressed in the developing OEs (dashed lines) of PEA-exposed or control fish at any time point examined (E). *dmbx1* is found in the developing CNS (F). A-E: Ventral views, anterior to the top of the page. F is a lateral view, anterior to the left. con= control; PEA= PEA-exposed. Scale bars: A (A,B)= 30μm; C (C,D)= 30μm; E= 50μm.


64h
her4
con
A
64h
her4
PEA
B
64h
crabp1a
con
C
64h
crabp1a
PEA
D
48h
dmbx1
E
48h
dmbx1
F

expressed throughout the embryo and is found in the basal part of the developing OE and in cells immediately outside of the epithelium from 48h-72h (Fig. A3A, arrow). Upon, PEA- exposure (Fig. A3B, arrow) there was a slight increase in the level of *her4* mRNA expression when compared to control (Fig. A3A) preparations run in parallel. This increase was not examined further because attention was put towards studying *otx2*. *crabp1a* is expressed in a small cluster of cells that are found immediately outside of the developing OE beginning at 56h (Fig. A3C, arrow). There was no change in the expression of *crabp1a* when animals were exposed to PEA (Fig. A3D, arrow). *dmbx1* was not expressed in the developing OE of control or PEA-exposed juveniles at any time point examined (Fig. A3E) but the probe did work as seen by expression of *dmbx1* in the CNS (Fig. A3F). These expression experiments identified two other genes that are expressed in *(her4)* or extremely close to *(crabp1a)* the developing OE and along with *otx2* served to confirm the microarray experiment as being able to identify genes that are expressed in the peripheral olfactory system.

Three attempts were made to repeat the microarray analysis but they were unsuccessful due to apparent problems with the microarray hybridization procedure. The results from the repeat arrays were not consistent with the first microarray and were very difficult to interpret because the majority of the up-regulated genes did not seem to make any logical sense. For example, there was a high proportion of genes involved in development of the vertebrate eye. This is an extremely unlikely result because RNA was only isolated from olfactory epithelia and the eyes of the fish were completely removed before the noses were isolated from the nasal capsules. Upon examination of the images of the microarrays (Fig. A4), which were sent to us by the microarray

Figure A4. Images of microarrays hybridized with RNA from PEA-imprinted and control OEs. A: Image representative of spotting on original microarray (October, 2003). Spots were of relatively equal size and hybridization was even throughout the spot (arrow). B: Image from repeat microarray from February, 2005. There was a high proportion of uneven spots (arrowhead) and spots that ran together (open arrow). C: Image of repeat microarray from March, 2005. Many of the spots were uneven and fragmented (open arrowhead).


A
B
C

facility at the Kimmel Cancer Center where the arrays were done (see Chapter three, methods), it was clear that the quality of the first array (which identified *otx2* and the other genes described above) was superior to the quality of the other arrays. Figure A4 shows representative images from the original array (Fig. A4A, October, 2003) and two of the three repeat arrays from February, 2005 (Fig. A4B) and March, 2005 (Fig. A4C). The spots throughout the original array were evenly hybridized and of relatively equal size (Fig. A4A, arrow). In contrast, there were a high proportion of spots on the February array that were even in the middle but had rings around the outside (Fig. A4B, arrowhead). There were also many spots that bled together (Fig. A4B, open arrow). The spots on the March array were extremely uneven and broken up (Fig. 4C, open arrowhead). The RNA for all experiments was examined on a formaldehyde gel before being sent to the microarray facility, which eliminated poor sample quality as a reason for the inconsistent array results. The inconsistency of the spotting in the repeat arrays indicates that there may have been problems with the hybridization of these arrays. The "reading" for each spot is taken as an average of the signal across all of the pixels within a spot so a high proportion of poorly hybridized, mis-shapen spots can lead to inconsistent, unreliable results (DM Lin, personal communication). The results obtained from the repeat arrays were difficult to interpret and not trustworthy and therefore not used in further analysis. At that point in time there were no more imprinted OEs available for RNA isolation so more microarray analysis was not an option. Recently, OEs from a new group of imprinted fish were isolated and these could be used for more microarray analysis in the future.

ORs were also present on the microarray. Table A2 shows all of the receptors examined between QRT-PCR and microarray analysis, whether or