Mouse Olfactory Sensory Neurons Express 10,000 Genes

NEERAJA SAMMETA, TUN-TZU YU, SOMA C. BOSE, AND TIMOTHY S. McCLINTOCK*

Department of Physiology, Cellular and Molecular Neuroscience of Sensory Systems Training Program, University of Kentucky, Lexington, Kentucky 40536-0298

Abstract

Olfactory epithelial cells from olfactory marker protein-green fluorescent protein (OMP-GFP) mice were separated by fluorescence-activated cell sorting into a GFP+ sample enriched in mature olfactory sensory neurons (OSNs) and a GFP− sample enriched in all other cells. GeneChip expression profiling of these samples provided a predictive measure of expression in OSNs. Validation tests comparing the ratio of GFP+/GFP− signal intensity against expression patterns from in situ hybridization for 189 mRNAs proved statistically significant and provided probabilities of expression in OSNs scaled according to the signal intensity ratios. These probabilities predict that, among 11,596 mRNAs detected in the GFP+ sample, more than 10,000 are expressed in OSNs. Transcripts and overrepresented categories of mRNAs detected in the GFP+ sample agreed with known properties of OSNs and predict additional properties. For example, ciliogenesis and spermatogenesis were overrepresented, consistent with similarities between OSN cilia and sperm flagella. Chromatin assembly mRNAs were expressed throughout the OSN cell lineage, consistent with the hypothesis that chromatin remodeling plays a role in OSN differentiation. We detected numerous signaling proteins and receptors, such as 30 nonchemosensory G-protein-coupled receptors, including the presynaptic glutamate receptor mGlur4 and the Wnt receptor Fzd3. The largest group of mRNAs, however, was the hundreds of transcriptional regulators that presumably determine the OSN phenotype. The absence of OMP protein in OMP-GFP mice had no detectable effect on mRNA abundance. Within limits prescribed by the nature of microarray data and the in situ hybridization validation, these data should be useful in directing further experiments on OSN function. J. Comp. Neurol. 502:1138–1156, 2007. © 2007 Wiley-Liss, Inc.

Indexing terms: chromatin assembly; cilia; neural differentiation; growth factor; cell adhesion; microarray

The nervous system is a complex cellular environment, largely because of the great diversity of types of neurons. This diversity is known from differences in position, morphology, neurochemistry, physiology, expression of marker genes, or a combination of these features. Although much is known about many types of neurons, a complete description of any particular phenotype has not been achieved. The foundation for phenotype for any cell is the set of genes it expresses. As exemplified by the functional genomics of single-celled organisms such as yeast, significant steps toward the complete phenotypic description of a cell can be made if the expressed genes are identified (Grunenfelder and Winzeler, 2002; Ideker et al., 2001; Panda et al., 2003). Knowing this set of genes explains the molecular underpinnings of known functions, predicts new functions, and defines the capabilities of the cell. This rationale stimulated us to determine whether it is possible to advance our understanding of the phenotype of a specific type of neuron by determining the mRNAs it expresses.

Only recently has it become possible to come close to the goal of identifying all genes expressed by a neuron. The
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Materials and Methods

Cell dissociation, FACS, and RNA isolation

Testing the effect of the absence of OMP on gene expression was done with mice at age P11 using three pooled samples from each genotype (N = 3), with three mice per pool. The M430v2.0 GeneChip was used to profile RNA in these samples, with 4.5 µg of RNA used from each sample. Hybridization, scanning, and data analysis were performed. The dissociation of cells from the olfactory epithelia of mice homozygous at the OMP-GFP locus and FACS procedures were as described previously (Yu et al., 2005), except that staining for LacZ expression was omitted, and cells from FACS were collected in fetal bovine serum before being concentrated by centrifugation and lysed in TRIReagent (Molecular Research Center Inc., Cincinnati, OH). In total, 11 postnatal day 11 (P11) pups from three different litters of three mothers were used to obtain 500,000 fluorescent cells (the GFP+ sample) and 2,500,000 nonfluorescent cells (the GFP− sample). Brains (minus olfactory bulbs) were collected from three of these OMP-GFP mice and pooled to provide a brain sample. Total RNA was isolated by the TRIReagent standard procedure supplied by the manufacturer.

GeneChip microarrays and bioinformatics

Expression profiling of the GFP+, GFP−, and brain samples used the M430A/B GeneChip set. Because the GFP+ sample was difficult to obtain and limited to 2.5 µg of RNA, a single GeneChip set was tested (N = 1), and only 2.5 µg of RNA of each sample was used. Our goal was to measure mRNA abundance for comparison with in situ
hybridization data, so, unlike most applications of microarray technology, no statistical testing for differences between the GFP\(^+\) sample and the GFP\(^-\) sample was desired or done. Instead, statistical analyses of the comparison between the GeneChip data, quantified as the ratio of signal intensity between the GFP\(^+\) and the GFP\(^-\) samples, and in situ hybridization data, categorized according to the cell types labeled, were carried out. First, ANOVA was used to test the hypothesis that mRNAs expressed in OSNs tended to have high GFP\(^+/\)GFP\(^-\) ratios. Then, hypotheses about the ability of ranges of GFP\(^+/\)GFP\(^-\) ratios to predict expression in OSNs were tested by using previously published in situ hybridization data (Yu et al., 2005). These data suggested that ratios \(\geq 1.3\) would be highly correlated with expression solely in OSNs and that ratios ranging from 1.2 to 0.5 would also include a substantial fraction of mRNAs expressed in OSNs, because many mRNAs are shared between OSNs and other neighboring cell types, and many mRNAs are expressed by both mature OSNs (enriched in the GFP\(^+\) sample) and immature OSNs (enriched in the GFP\(^-\) sample). The comparison between microarray data and in situ hybridization data provides a statistical basis for predicting expressed in OSNs, but, because in situ hybridization is less sensitive than GeneChip expression profiling arrays, we are unable to assess reliably probe sets with low signal intensities. We therefore used not only the Affymetrix MAS5.0 software absence call to eliminate questionable probe sets but also set a criterion of 15\% of the mean signal for detection. This threshold derived from our empirical observation that probe sets exceeding it represented mRNAs that could usually be detected by in situ hybridization. Microarray data have been deposited at Gene Expression Omnibus (accession Nos. GSE4927 and GSE4915).

Statistically overrepresented and underrepresented groups of functionally related genes were identified by using EASE, GenMAPP, or DAVID 2006 (Dahlquist et al., 2002; Dennis et al., 2003; Hoxack et al., 2003). These programs use controlled vocabularies, usually that of the Gene Ontology Consortium, to recognize genes containing the same or related functional annotation. In Gene Ontology, this annotation is organized into categories: Biological process, molecular function, and cellular compartment. All three programs use statistical tests such as Fisher’s exact test to detect overrepresented categories. Whenever possible, we also used false discovery rate corrections to reach a corrected \(\alpha\) level of \(P < 0.05\). In the case of DAVID 2006 analyses, we used an \(\alpha\) level of \(P < 0.01\). The grouping of genes by function was then further augmented by literature searches on the functions of hundreds of gene products.

**In situ hybridization**

Wild-type C57Bl/6J mice of both sexes, ages P21–P28, were used for two reasons. They provided an additional control for changes that might result from the OMP-GFP genotype, and they help to confirm that the microarray expression patterns obtained at P11 were maintained into maturity. The selection of mRNAs for in situ hybridization was based on numerous criteria. Twenty-two were selected based solely on signal intensity differences between the GFP\(^+\) and GFP\(^-\) samples; the remainder were selected because they expressed proteins with functions of potential significance to OSNs or other cell types in the olfactory epithelium (Supplementary Table 1). In situ hybridizations were performed as described previously (Shetty et al., 2005; Yu et al., 2005). In brief, dissected dorsal regions of the snout and anterior cranium from mice transcardially perfused and fixed in 4\% paraformaldehyde were cryoprotected, stored at \(-80\)°C, and cut into 10–15-μm coronal sections on a cryostat. One to four digoxigenin-labeled riboprobes, typically about 500 bp in length, were prepared for each mRNA and hybridized in 50\% formamide in 10 mM Tris-HCl (pH 8.0), 10\% dextran sulfate, 1× Denhardt’s solution, 600 mM NaCl, 0.25\% SDS, 1 mM EDTA, and 200 μg/ml yeast tRNA at 65°C (1 ng/μl per riboprobe) on cryosections mounted on slides. Detection used an alkaline phosphatase-conjugated antibody to digoxigenin and hydrolysis of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine. Sense and antisense probes were always tested simultaneously, and the sense controls were invariably negative. Antisense labeling that was consistent throughout layers corresponding to cell types and that was noticeably darker than sense labeling and background labeling of acellular locations was considered a positive signal. Digital wide-field images were obtained with a Spot 2e camera on a Nikon Diaphot 300 inverted microscope. Images were processed in Adobe Photoshop by adjusting size, brightness, and contrast and by using the dodging tool to eliminate shadows in open areas of some images to improve the consistency of illumination. They were then combined and labeled in Deneba Canvas.

**RESULTS**

**Absence of OMP had no effect on mRNA abundance**

To determine whether OMP-GFP mice are suitable models for assessing gene expression in OSNs, we had to test whether OMP participates in regulating mRNA abundance. OMP is an enigmatic protein expressed almost exclusively in mature OSNs, arguing that its function is specially suited to the needs of OSNs. None of the properties of OMP suggests any direct role in regulating gene expression in OSNs (Behrens et al., 2003; Buiakova et al., 2005; Carr et al., 1998; Farbman and Ezeh, 2000; Ivic et al., 2000; Koo et al., 2004, 2005), but to confirm that gene expression in the olfactory epithelia of mice lacking OMP is indistinguishable from that in mice expressing OMP, we compared homozygous and heterozygous OMP-GFP mice. By using Affymetrix M430v2.0 GeneChips, we discovered no statistically significant differences in mRNA abundance between these two genotypes. Even the OMP mRNA from the targeted gene locus, which lacks the OMP coding region but retains the 3'-untranslated regions detected by its probe set on the GeneChip, did not differ. To help confirm this result, we performed in situ hybridization for two of the three mRNAs that showed the largest differences, Cacna2d1 and Mtmr7 (Fig. 1). As predicted by the absence of significant differences in the microarray data, the labeling intensities and expression patterns for these mRNAs showed little difference between OMP-GFP\(^-\)/OMP-GFP\(^+/\) mice. These results argue that OMP-GFP mice are valid models for assessing gene expression patterns in the olfactory epithelium even when OMP protein is completely lacking.
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Comparisons among the three samples identified 730 probe sets that were detected in the GFP⁻ sample but not in the GFP⁺ sample or in brain. In the reciprocal comparison, 2,107 probe sets were detected only in the GFP⁻ sample. The probe sets with signals specific for the GFP⁺ sample represent at least 615 distinct mRNAs, with 508 of these having signal intensities greater than 15% of the mean signal. Similarly, the probe sets specific to the GFP⁻ sample represent at least 1,721 mRNAs, with 1,384 of these having signal intensities greater than 15% of the mean signal. The cellular complexity of the GFP⁻ sample impairs our ability to make interesting predictions about function, so we have concentrated our efforts on analyzing the GFP⁺ sample.

To test the prediction that the mRNAs specific to the GFP⁺ sample were expressed only in OSNs, we selected 16 mRNAs whose probe set signal intensities spanned the breadth of signal intensity in the microarray data and tested them by in situ hybridization. All that gave detectable signals labeled the mature OSN layer, or the mature and immature OSN layers, of the olfactory epithelium. Examples of these results are shown in Figure 2A–F. To test the prediction that the mRNAs detected specifically in the GFP⁺ sample were expressed primarily in the cells neighboring mature OSNs, we performed a similar analysis for six mRNAs. All of these mRNAs were detected exclusively in cells outside the mature OSN layer of the epithelium (Fig. 2G–L).

Large numbers of genes are already known to be expressed only in OSNs within the olfactory epithelium, but, surprisingly, just 27 of them were among the 615 mRNAs detected only in the GFP⁺ sample, and 17 of these were odorant receptors. The others were OMP, Adcy3, Bmp6, Ebf4, Nqo1, Hmox1, Nfe2l2, and Pde1c. This finding predicts that most known OSN-specific mRNAs are found in both mature and immature OSNs. Indeed, when we checked 51 such mRNAs, all of them were detected in the GFP⁺ and GFP⁻ samples, consistent with expression in both mature and immature OSNs, respectively. These findings argued that specificity to the GFP⁺ sample was not necessary to predict expression in OSNs accurately.

Transcripts enriched in mature OSNs

Separation of mature OSNs from neighboring cells

Separating GFP⁺ and GFP⁻ cells from the olfactory epithelia of OMP-GFP mice should produce a population of cells highly enriched in mature OSNs (GFP⁺) and a population enriched in all other cell types in the epithelium (GFP⁻). The GeneChip expression profiles of these two cell populations reflected this separation. The OMP-GFP mRNA from the OMP locus, detected via the 3' untranslated region undisturbed by the deletion of the coding region, was detected in the GFP⁺ samples but not in the GFP⁻ sample. Another mRNA specific to mature OSNs, Umodl1 (N8), was 33-fold enriched in the GFP⁺ sample (Yu et al., 2005). Similarly, inspection of the data for specific markers of other cell types indicated stronger enrichment for these cells in the GFP⁺ sample. Ngn1 (immediate neuronal precursors), Krt1-14 (horizontal basal cells), and Reg3g (respiratory epithelium) were absent in the GFP⁺ sample but present in the GFP⁻ sample. The single GAP-43 (immature OSNs) probe set on the array showed twofold enrichment in the GFP⁻ sample. The marker of transit amplifying progenitor cells Ascl1 (Mash1) was represented by two probe sets. They were enriched tenfold and >24-fold in the GFP⁻ sample. The sustentacular cell markers Cbr2 and Pax6 were enriched twofold and threefold, respectively, in the GFP⁻ sample (Yu et al., 2005). These distributions show that the FACs samples had significant separation of mature OSNs from the other cell types in the olfactory epithelium.

Fig. 1. In situ hybridization for two mRNAs that differed most between the microarray results for OMP⁻ and OMP⁺ olfactory epithelia. A,B: Cacna2d1 (calcium channel, voltage dependent, alpha2/delta subunit 1) shows similar expression patterns and labeling intensity in both genotypes. C,D: Mtmr7 (myotubularin related protein 7) also shows little difference in expression between the genotypes. E: A guide to the cell body layers of the pseudostratified olfactory epithelium and the locations of the basement membrane (basal lamina) and lamina propria (stroma) in a mouse aged 3 weeks. In situ hybridization for OMP labels all mature OSNs. The sustentacular cell layer above the OSNs is unlabeled. Immature OSNs lie immediately below the mature OSNs and form an irregular boundary with them. Immediately above the basement membrane (basal lamina) are basal cells, some of which are labeled with an asterisk. Not identified in this image are Bowman’s glands that sit just below the basement membrane and the associated duct cells that extend apically across the epithelium (but see in situ hybridization for Elf5, Fig. 2G). Apical is upward in these and all subsequent images. Scale bars = 25 μm.
and that mere enrichment in the GFP\(^+\) sample would suffice. In situ hybridization for 52 mRNAs enriched two-fold or more in the GFP\(^+\) sample revealed that 35 of them were detected only in OSNs. The other 17 gave signals most strongly in OSNs but were also detected in other cell types.

**GFP\(^+\)/GFP\(^-\) signal intensity ratios predicted expression in OSNs**

The analysis of OSN-enriched mRNAs agrees with the expectation that many genes expressed by OSNs are shared with many other cell types. However, we should still be able to use comparison of the GFP\(^+\) and GFP\(^-\) samples as one element of an approach to rigorously predict mRNAs found in OSNs. To evaluate this idea, we tested two hypotheses by comparing the GFP\(^+\)/GFP\(^-\) signal intensity ratios determined from the microarray data with cell type determinations done by in situ hybridization for 189 mRNAs (Fig. 3). First, mRNAs expressed in OSNs should have higher GFP\(^+\)/GFP\(^-\) ratios than mRNAs expressed in neighboring cells. This proved true ($F = 2.478$, $P = 0.03$). Second, we used previously published in situ hybridization data (Yu et al., 2005) to establish the hypothesis that a GFP\(^+\)/GFP\(^-\) ratio $\geq 1.3$ would accurately predict expression in OSNs. This also proved true ($x^2 = 68.6$, $P < 0.0001$). Even though most mRNAs expressed in OSNs were separated into both GFP\(^+\) and GFP\(^-\) samples because of the specificity of the OMP gene locus for mature OSNs, a high GFP\(^+\)/GFP\(^-\) ratio accurately predicted expression in OSNs. In fact, among the 108 mRNAs with ratios $\geq 1.3$ tested, only one mRNA failed the prediction.

**Genes expressed in OSNs number more than 10,000**

For the GFP\(^+\) sample, we detected positive signals on 19,861 probe sets. With the caveat that unannotated genes make the calculation an underestimate, these 19,861 probe sets represent at least 11,596 different genes. Because it contains more cell types, we predicted that the numbers in the GFP\(^-\) sample would be larger. Indeed, we detected 22,729 probe sets, representing mRNAs from at least 13,272 genes, with positive signals in the GFP\(^-\) sample. The total number of mRNA species detected in the combined microarray data from the GFP\(^+\) and GFP\(^-\) samples was 14,104. With sufficient in situ hybridization data, a reasonable estimate of how many of these are expressed in OSNs should be possible.
As described in the preceding section, a GFP+/GFP− ratio ≥ 1.3 was a highly accurate predictor of expression in OSNs. This measure is valid for mRNA species that can be detected by in situ hybridization, which we determined empirically to be signals above 150 (15% of the mean signal of ~1,000 in this experiment). In total, 4,740 mRNAs meet this criterion. The frequency of error in this prediction can be estimated by our in situ hybridization data as 1 in 108, predicting that 4,696 of these mRNAs are expressed in OSNs.

Many genes expressed by OSNs could not be expected to meet such stringent criteria, of course. Many mRNAs are common to many or all cell types and therefore fail the ratio criterion even though they are present in OSNs. These mRNAs should have intermediate GFP+/GFP− ratios, and many of them should exceed the signal intensity threshold of 150. In addition, even most mRNAs enriched in OSNs are expressed in both mature and immature OSNs and therefore should have an intermediate ratio because the FACS separated these two developmental stages into different samples. Checking a group of mRNAs previously identified in OSNs (Yu et al., 2005) showed that several had GFP+/GFP− ratios between 1.2 and 0.5. Our data identified 10,947 mRNAs with GFP− signals above 150 and GFP+/GFP− ratios above 0.5. Among these, 6,107 had GFP+/GFP− ratios between 1.2 and 0.5. Our in situ hybridization data included 37 of these mRNAs. Among these 37 mRNAs, 34 were detected in the OSN layer (nine of the 34 were also detected in other cell types). This fraction yielded an estimate of 5,611 additional mRNAs expressed by OSNs. To be more rigorous, however, we generated a 95% confidence interval for this comparison. The lower end of the 95% confidence interval, 83%, predicts that 5,068 of this pool of 6,107 mRNAs are expressed in OSNs.

In addition, OSNs should contain rare mRNAs that we detected only at levels below the 15% of mean signal criterion. Except for cases of low signals resulting from poor performance of probe sets, these mRNAs are of low abundance and would be difficult or impossible to detect by in situ hybridization but are nevertheless present in OSNs. The GFP+/GFP− ratio criterion can be used to predict at least a subset of these low-abundance mRNAs that are present at higher levels in mature OSNs than neighboring cells. Our data contained 367 mRNAs that have GFP+/GFP− ratios ≥ 1.3 and GFP− signals below 150. The total nonredundant estimate of mRNAs detected in OSNs is therefore 4,740 + 5,611 + 367 = 10,718. A more conservative calculation of 4,696 + 5,068 + 364 = 10,128 also yields more than 10,000 genes (Table 1).

### Biological processes over- and underrepresented in mature OSNs

The prediction of genes expressed in OSNs provided information not only on individual gene products but also about which biological processes and pathways are potentially active in OSNs. We first pursued this analysis for the 508 mRNAs specific to the GFP− sample and discovered three clusters of overrepresented Gene Ontology categories: smell, ion transport, and cilium/flagella. The overrepresentation of smell in the mRNAs specific to the GFP− sample was due to the detection of numerous mRNAs known or expected to be expressed only by OSNs, primarily 26 odorant receptors and components of the olfactory transduction cascade. The category of ion transport was due to a group of ion channel subunits detected only in the GFP− sample: Scn5a (Nav1.5), Kcnc4 (Kv3.4), Kcnk3 (Kv9.3), Kcnk16, and Kcnk10 (1700024D23Rik). As this analysis predicts, when we tested which cells expressed four of these mRNAs, all were found in OSNs (Fig. 2C–F). The categories of cilia and flagella represent a defining property of mature OSNs and are discussed in detail below.

To look more broadly at functions enriched in OSNs, we performed functional bioinformatics analysis of the 11,596 mRNAs detected in the GFP− sample and among the 2,682 mRNAs enriched twofold in the GFP− sample. These analyses identified additional overrepresented groups of functionally related gene products (Table 2). Some, such as metabolism, protein transport, ribosome biogenesis, and RNA processing, are ubiquitous functions. The individual mRNAs in these categories were indeed nearly all common mRNAs that support basic cellular functions. Transcriptional regulation, kinases and phosphatases, cell adhesion, calcium binding, tetraoctapeptide region (TPR) repeat proteins, WD repeat proteins, and zinc finger protein categories appeared to include both groups of common mRNAs and mRNAs potentially important for functions more restricted to OSNs. The remaining

| GFP+/GFP− ratio | Microarray signal | mRNAs in pool | Confidence (%) | No. predicted in OSNs |
|-----------------|------------------|---------------|---------------|----------------------|
| ≥ 1.3           | ≥ 150            | 4,740         | > 99 (P < 0.0001) | 4,696                |
| 1.2–0.5         | ≥ 150            | 6,107         | > 83 (P < 0.05)  | 5,068                |
| ≥ 1.3           | ≤ 150            | 367           | > 99 (unknown)  | 364                  |

*Confidence, the likelihood that any given mRNA picked from the candidate pool is expressed in OSNs; parentheses, the statistical probability associated with this likelihood estimation.
categories, however, represent processes and functions more relevant to the special properties of OSNs. Chromatin assembly and remodeling have been proposed to be especially critical for gene expression in the OSN cell lineage (Shetty et al., 2005). Ciliogenesis, which is related to spermatogenesis via the shared properties of cilia and flagella, is required for OSN function. Ion channels are necessary for odor detection and excitability. Synaptic vesicles are necessary for synaptic transmission to second-order neurons in the olfactory bulb. Apoptosis is necessary to eliminate damaged OSNs. To confirm that potentially significant genes in several of these categories are expressed in OSNs, we performed additional in situ hybridization studies.

### Chromatin assembly and remodeling in the OSN lineage

Our previous study of mRNA abundance changes induced by ablation of the olfactory bulb indicated that OSNs are enriched in mRNAs involved in gene silencing and chromatin assembly (Shetty et al., 2005), consistent with the identification of chromatin assembly as an overrepresented category in the GFP* sample. Several different types of chromatin remodeling proteins were enriched in the GFP* sample, but the most prominent functional groups were components of two types of complexes that control gene transcription via chromatin structure: the SWI/SNF complexes and the Polycomb complexes (Table 3, Supplementary Table 2A). We confirmed expression in OSNs of several of these mRNAs (Fig. 4).

SWI/SNF complexes regulate transcription by modifying DNA-histone contacts, activities that may also be used in DNA repair and replication (Martens and Winston, 2003). Our microarray data indicated expression of numerous SWI/SNF complex genes in the olfactory epithelium. We performed in situ hybridization to determine the expression pattern of several of these mRNAs (Table 2). We confirmed expression in OSNs, we performed additional in situ hybridization studies.

### TABLE 2. Significantly Overrepresented Gene Ontology Categories Among the mRNAs Detected in the GFP* Sample (Purified Mature OSNs)*

| Gene | Ratio | Signal | Description |
|------|-------|--------|-------------|
| Chx2 | 1.160 | 0.6 | OSN, Polycomb, PRC1 |
| Chx4 | 9.159 | 2.6 | OSN, Polycomb, PRC1 |
| Chx8 | 787.3 | 4.5 | OSN, Polycomb, PRC1 |
| Peg2 | 192.4 | 1.3 | OSN, Polycomb, PRC1 |
| Bmi1 | 1.453 | 1.2 | OSN, Polycomb, PRC1 |
| Peg6 | 637.7 | 0.8 | OSN, Polycomb, PRC1 |
| Phc2 | 888.9 | 0.6 | Basal, OSN, Polycomb, PRC1 |
| Rnf2 | 1,009 | 0.8 | Basal, OSN, Polycomb, PRC1 |
| Epc1 | 1,969 | 1.2 | Basal, OSN, Polycomb, PRC2 |
| EzH1 | 745 | 1.6 | Basal, OSN, Polycomb, PRC2 |
| EzH2 | 380 | 0.2 | Basal, OSN, Polycomb, PRC2 |
| Peg1 | 346 | 1.5 | OSN, Polycomb, PRC2 |
| Yy1 | 1,764 | 1.7 | OSN, Polycomb, PRC2 |
| Arid1a | 2,439 | 0.8 | OSN, SW/SNF complex |
| Smarcb1 | 831 | 0.7 | Basal, OSN, SW/SNF complex |
| Smarcb2 | 236 | 0.9 | Basal, OSN, SW/SNF complex |
| Cbx8 | 787 | 4.5 | OSN, Polycomb, PRC1 |
| Cbx4 | 1,160 | 0.6 | OSN, Polycomb, PRC1 |
| Cbx2 | 1,027 | 0.5 | Basal, OSN, SW/SNF complex |

*Includes biological process, function, or cellular compartment categories. Subcategories are indented. Statistical analyses were performed by using EASE, GenMAPP, and DAVID 2.0. To simplify the table, related categories were combined. Parentheses, number of categories combined.

Several other types of chromatin remodeling mRNAs were detected in our expression profiles. Nuclear remodeling and deacetylase (NuRD) complex mRNAs were detected, and all had GFP*/GFP− ratios <0.8, suggesting expression primarily in immature OSNs or nonneural cells. We have not yet tested this prediction. However, we did determine the expression patterns of several other types of chromatin remodeling mRNAs. Ttk1, a highly conserved kinase that is involved in chromatin condensation, chromatin assembly, and DNA damage checkpoints (Groth et al., 2003; Sunavala-Dossabhoy et al., 2003), was expressed in OSNs (Fig. 4O). Setd7, a methyl transferase associated with transcriptional repression via methylation of histone H4 (Karachentsev et al., 2005), was also expressed in OSNs (Fig. 4P). Chromatin remodeling complexes that promote repression often associate with histone deacetylases. We detected expression of several histone deacetylases, including HDACs 1–6 and 8–11 in the GFP* sample. The GFP*/GFP− ratios of HDAC mRNAs indicate that Hdac4, Hdac9, and Hdac11 are expressed primarily by OSNs, but we have not yet tested this prediction.

### Ciliogenesis and support of cilia in OSNs

The overrepresentation of cilia and flagella mRNAs in the GFP* sample was consistent with the developmental timing of ciliogenesis in OSNs, which happens as OSNs become mature (Cuschieri and Bannister, 1975). We...
would therefore expect the GFP+/GFP– ratios for these mRNAs to be high, and indeed they were (Table 4, Supplementary Table 2B). We tested several of these mRNAs by in situ hybridization, and in every case they were detected in OSNs (Fig. 5). They included mRNAs for several proteins found in the sperm flagella and its fibrous sheath or related to proteins found there, such as Ropn11, Gas8, Fsp1, Spag16, and Spa17 (Eddy et al., 2003). Other
ers encode components of the basal body or ciliary rootlet, including the Bardet-Biedl syndrome (Bbs) proteins and Crocc (rootletin), or in the case of Clgn are potentially involved in ER transport of ciliary proteins in sperm (Kulaga et al., 2004; Nishimura et al., 2004). Gmcl1, Bcl6, Foxj1, Rfx4, and Rfx3 are all transcription factors known or suspected to be critical for the formation or function of sperm or other ciliated cells, so their expression in OSNs suggests the hypothesis that they contribute to the major shared feature of such cells, the cilium or flagellum (Bon naïve et al., 2004; Kimura et al., 2003; Kojima et al., 2001; Maekawa et al., 2004).

**Growth factors and their receptors in OSNs**

Even though signal transduction was not an overrepresented biological process in the GFP/H11001 sample, except for categories of kinases and phosphatases, OSNs are known to respond to signals besides odors. They receive trophic support from the bulb (Schwob et al., 1992); they are known to express Tgfr2, Igf1r, and Fgfr1; and the Egfr is expressed in a small subset of OSNs (Getchell et al., 2002a; Salehi-Ashtiani and Farbman, 1996; Suzuki and Takeda, 2002). Our results confirm and extend these findings. The GFP/H11001 sample contained positive signals for at least 16 growth factor receptor mRNAs and 14 growth factor mRNAs (Supplementary Table 2C).

There were eight different bone morphogenic protein (BMP)/transforming growth factor-β (TGFβ) family receptor mRNAs detected, indicating that OSNs closely monitor this large superfamily of growth factors. We confirmed expression of Bmpr1a, Tgfr2, and Acrv1 mRNAs (Fig. 6A–C) plus the downstream signaling components Smad2 and Tieg1 (not shown) in OSNs by in situ hybridization. OSNs also express mRNAs that encode regulators of BMP signaling. We found that Tws2l, Ltbp2, and Bmpr mRNAs were expressed in OSNs (Fig. 6D,E, Table 5). These mRNAs encode secreted proteins that interact with BMPs and regulate their activity at their targets (Moser et al., 2003; Zakin and De Robertis, 2004).

OSNs are capable of detecting fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), and platelet-derived growth factors (PDGFs). Our data indicate that, like many other neurons, OSNs express Fgfr2 and Fgfr4 (Fuhrmann et al., 1999). We used in situ hybridization to confirm expression of Pdgfrb, Pdgfrl, and Igf1r in OSNs, whereas Igf2r mRNA was detected in OSNs and in subsets of basal cells (Fig. 6F–I, M). The GFP+ sample contained a surprising number of growth factor mRNAs. OSNs are already known to express several signaling proteins, including Gdnf, Cntf, Fgf2, Bmp4, Bmp6, Bmp7, and GDF11 (Buckland and Cunningham, 1999; Goldstein et al., 1997; Hsu et al., 2001; Peretto et al., 2002; Shou et al., 2000; Wu et al., 2003). We detected several additional growth factors

| Gene symbol | GFP+ signal | GFP+/− ratio | ISH Function |
|-------------|-------------|--------------|--------------|
| Bbs2        | 1,043.1     | 3.4          | OSN Flagellar function |
| Crocc       | 515.7       | 43.7         | OSN Flagellar function |
| Fgfr1       | 258.5       | 2.9          | OSN Flagellar function |
| Ropn1l      | 157.6       | 8.6          | OSN Flagellar function |
| Spag16      | 702.6       | 7.1          | OSN Flagellar function |
| Clgn        | 27,503.5    | 7.9          | OSN Flagellar transport |
| Rfx1        | 2,319.2     | 2.8          | OSN Flagellar transport |
| Patl1       | 1,582.9     | 21.1         | OSN Sperm function |
| Spa17       | 1,981.7     | 3.7          | OSN Sperm function |
| Dna1p13     | 3,251.1     | 26.7         | OSN Spermatogenesis |
| Gash        | 409.0       | 2.8          | OSN Spermatogenesis |
| Gmcl1       | 2,582.3     | 1.3          | OSN Spermatogenesis |
| Ndrg3       | 2770        | 5.1          | OSN Spermatogenesis |
| Tbp1l       | 1,134.6     | 1.2          | OSN Spermatogenesis |

1ISH, in situ hybridization; basal, basal cells.

**Fig. 5. In situ hybridization for ciliogenesis and spermatogenesis mRNAs, which were overrepresented biological process categories in the GFP+ sample, consistently showed expression in OSNs.**

A: Ropn1l (ropporin 1-like). B: Spag16 (sperm associated antigen 16). C: Gas8 (growth arrest specific 8). D: Bbs2 (Bardet-Biedl syndrome 2 homolog). E: Crocc (ciliary rootlet coiled-coil, rootletin). F: Gmcl1 (germ cell-less homolog). G: Foxj1 (forkhead box J1). H: Rfx3 (regulatory factor X, 3). I: Fsip1 (fibrous sheath-interacting protein 1). J: Hybridization of sense riboprobe of Fsip1. Scale bars = 25 μm.
in the GFP⁺ sample, including Fgf2, Fgf9, Bmp6, Hdgf, and Fgf12 (Table 5, Supplementary Table S2). Our in situ hybridization data indicate that Hdgf is expressed in OSNs but even more strongly in a subset of basal cells (Fig. 6J).

### G-protein-coupled receptors in mature OSNs

Several of the signals known to act on OSNs are likely to activate G-protein-coupled receptors (GPCRs; Hegg and Lucero, 2004; Koster et al., 1999; Levasseur et al., 2004). In agreement with these observations, we detected the D2 dopamine receptor and the 1A subtype of the arginine-vasopression receptor in the GFP⁺ sample. In total, we detected 26 odorant receptors and 56 GPCR mRNAs that are not chemosensory receptors in the GFP⁺ sample, with 30 of them having sufficiently large signals or GFP⁺/GFP⁻ ratios to predict their expression in OSNs (Table 6, Supplementary Table 2D). We selected a subset of these GPCR mRNAs and attempted to verify their expression in OSNs. As shown previously, Drd2 was expressed by OSNs (Fig. 7A). OSNs are glutamatergic, indicating that they would express presynaptic metabotropic glutamate receptors. We confirmed expression of mGlur4 in OSNs (Fig. 7B). We also confirmed expression in OSNs for the somatostatin receptor Sstr4, the serotonin receptor Htr1b, the Frizzled family receptor Fzd3, the progestin
receptor Paqr8, the latrophilin receptor Lphn3, and three orphan GPCR mRNAs: Gpr37, Gpr158, and Gprc5c (Fig. 7C–H, Table 6). We tested three of the odorant receptor mRNAs that had the highest GFP/H11001 signal intensities: Olfr627, Olfr870, and Olfr112. Their expression was typical of odorant receptors, being expressed in scattered OSNs within restricted regions (zones) of the olfactory epithelium (data not shown).

Cell adhesion and cell migration mRNAs in OSNs

Our analyses indicated that cell adhesion was overrepresented in the GFP+ sample. Mature OSNs must maintain contact with several types of neighboring cells, both at the somatodendritic level in the olfactory epithelium proper and at their axons in the underlying stroma and olfactory bulb. Maintaining these neurites and their contacts, plus the need to move signals up and down the neurites, requires sets of specialized proteins. In addition, immature neurons have to grow axons across several tissue layers to reach the glomeruli, so they probably have to express a distinct set of cell adhesion and cell migration genes. In fact, we already have evidence of this from expression profiling of the response to ablation of the olfactory bulb (Shetty et al., 2005). Consistent with these expectations, the cell adhesion molecules and related mRNAs detected in the GFP+ sample tended to fall into two groups according to whether they are more abundant in the GFP+ or GFP− samples (Supplementary Table 2E).

The data indicate that mature OSNs express numerous cell adhesion molecules and several junctional complex gene products. We confirmed expression in OSNs of the cell adhesion molecules Dscam, Tcam, Nrnx1, Ncam1, and Ncam2; the glial receptor Astn1; and the junctional complex genes Arvfc, Dst, and Ssx2ip (Table 7, Fig. 8A–F,H). We also confirmed that low GFP+/GFP− ratios of Alcam (Fig. 8G) and Chl1 (Shetty et al., 2005) indicate expression in immature OSNs and basal cells. In contrast, the data indicate that mature OSNs express very few extracellular matrix components, the ones detected being mostly collagen, and probably have low amounts of mRNA for those they do not express (data not shown). Many of the cell adhe-

| Gene symbol | GFP signal | GFP+/− ratio | ISH |
|-------------|------------|--------------|-----|
| Tgfbr1      | 1,745.1    | 0.5          | Basal, OSN | Receptor |
| Igf2r       | 332.8      | 0.7          | Basal, OSN | Receptor |
| Igf1        | 1,155.9    | 0.9          | OSN | Receptor |
| Acrv1       | 241.3      | 1.2          | OSN | Receptor |
| Bmpr1a      | 266.7      | 1.5          | OSN | Receptor |
| Pdrg6l      | 836.2      | 3.7          | OSN | Receptor |
| Hdgf        | 911.5      | 0.5          | Basal, OSN | Signal |
| Fpf12       | 2,521.5    | 9.0          | OSN | Signal |
| Ttg1        | 1,544.1    | 1.3          | OSN | Regulator |
| Lzhp2       | 289.7      | 2.5          | OSN | Regulator |
| Bipper      | 714        | 2.7          | OSN | Regulator |

1Igfbp, which is expressed in OSNs (Fig. 6), is absent from this table because its probe set gave no signal for the GFP+ sample. ISH, in situ hybridization; basal, basal cells of the olfactory epithelium.

| Gene symbol | GFP signal | GFP+/− ratio | ISH |
|-------------|------------|--------------|-----|
| Lphn3       | 2,175.8    | 1.4          | OSN |
| Drd2        | 1,838.7    | 25.0         | OSN |
| Paqr8       | 1,321.3    | 2.7          | OSN |
| Gprc5c      | 1,028.1    | 2.5          | OSN |
| Gpr158      | 877.3      | 2.1          | OSN |
| Sstr4       | 728.1      | 1.1          | OSN |
| Gm1         | 686.7      | 1.2          | OSN |
| Fzd3        | 682.9      | 1.8          | OSN |
| Htr1b       | 659.8      | 1.4          | OSN |
| Gpr687      | 618.2      | 2.7          | OSN |

1ISH, in situ hybridization.
sion and extracellular matrix receptors detected in the GFP$^+$ sample had low GFP$^+$/GFP$^-$ ratios, indicating higher expression in other cell types or, alternatively, mRNAs more abundant in immature than in mature OSNs.

Transcriptional regulators in OSNs

We detected hundreds of mRNAs in the GFP$^+$ sample whose gene annotation indicated that they play some role in regulating gene transcription. Eliminating early components of signaling pathways and other proteins only peripherally involved in the regulation of RNA polymerases reduced the list to 438 genes that are known to encode proximal regulators of transcription or are potentially involved in transcription because of homology to known transcription factors. Among these mRNAs, 268 either have GFP$^+$/GFP$^-$ ratios $\geq 1.3$ or rank in the top 50% in signal intensity in the GFP$^+$ sample (Supplementary Table 3). We predict that these 268 genes are expressed in OSNs, and a significant number of the remaining 173 genes are probably also expressed in OSNs. We confirmed these predictions for some mRNAs via in situ hybridization (Table 8, Fig. 9).

DISCUSSION

This project has several outcomes that improve our capacity to understand the OSN phenotype. We identified 10,847 mRNAs that were sufficiently abundant (>15% of the mean signal) to be certain of their presence in our RNA samples and were able to assign to each of them a probability of expression in OSNs, yielding the calculation that more than 10,000 genes are expressed by OSNs. The identities of many of these genes correlate with known properties of OSNs and thereby predict gene products responsible for recognized phenotypic features. Transcription factors associated with ciliogenesis and stress responses, ion channels that support axonal excitability, structural proteins of cilia/flagella, and receptors for several signals to which OSNs respond are just a few examples. Even though the majority of mRNAs detected in the OSN-enriched sample encode proteins of unknown or uncertain function, these data predict many new or underappreciated aspects of the OSN phenotype. For example, the data strengthen the hypothesis that chromatin remodeling is very important to the olfactory epithelium, probably as a mechanism to control the changes in gene expression that drive differentiation of OSNs. Another example is the diversity of protein signals that OSNs appear to produce and the even greater diversity of receptors, especially GPCRs, that OSNs appear to express.
Limitations on the microarray data

Generating a signal intensity ratio between the GFP\(^+\) and the GFP\(^-\) samples for each mRNA and then using in situ hybridization to test predicted expression patterns provided a statistical basis for generating probability estimates for whether each mRNA was expressed in OSNs. This approach required large numbers of in situ hybridizations, but, by bringing to bear a combination of two independent measures in a way that allowed statistical discrimination, it overcame several potential problems. First, it allowed statistical confirmation that, within limits set by the sensitivity of in situ hybridization, the microarray data were sufficiently reliable to reflect the cell-type separation achieved by FACS. Second, it trumped deficits in cellular purity in the FACS, which can be difficult to achieve completely. Third, it relieved uncertainty about the degree of mRNA purity, which may differ from cellular purity. The purified cells came directly from a brief tissue dissociation procedure and were processed rapidly to isolate RNA, so dissociated OSNs could have carried fragments of neighboring cells along with them, and these fragments might have contained mRNAs. Lengthening the procedure to try to clean the cells further increases the chance of mRNA abundance changes, something we were unwilling to risk. Fourth, it allowed us to go beyond both simple presence/absence criteria and direct statistical comparison of the GFP\(^+\) and GFP\(^-\) samples, particularly important in this situation because most genes expressed by OSNs are expressed in both mature and immature OSNs and hence were present in both the GFP\(^+\) and the GFP\(^-\) samples.

In addition to these advantages, being cognizant of the limitations of our approach is critical for the proper use and interpretation of the data. First, the microarray data are only predictive, and these predictions are constrained by the extent of independent validation done by in situ hybridization. In essence, the predictions are valid for microarray signals that reach levels detectable by in situ hybridization. Second, although GeneChip expression profiling arrays are sufficiently accurate that technical replication is not necessary, more biological replicates would have improved the precision of our measures of mRNA abundance. This limitation further emphasizes the importance of the in situ hybridization data. The significant correlation between the microarray data and the in situ hybridization data argue that the use of single pooled samples was sufficient to measure mRNAs abundant enough to be detected by in situ hybridization. Low-abundance mRNAs lie beyond our ability to make good predictions, primarily because our approach could not independently validate them as a group but also because there is more uncertainty in the microarray measurement of their abundance. Third, microarray data are not reliable indicators of absent mRNAs, so by themselves the microarray data cannot be used to claim a gene is not expressed. Finally, our approach generates only probabilities of expression in OSNs, so a fraction of the predictions will be wrong. Even the list of mRNAs with GFP\(^+\)/GFP\(^-\) signal intensity ratios \(>1.3\) does not predict expression in OSNs with complete accuracy and therefore contains a few false positives. Fortunately, our approach estimates the expected frequency of these errors. Still, proving exactly which of the 11,596 mRNAs detected in the GFP\(^+\) sample are the more than 10,000 that we calculate are expressed in OSNs can be accomplished only via independent experiments.

The number of genes expressed by distinct cell types may be similar

Our data support the conclusion that OSNs express more than 10,000 genes. Uncertainty about the exact number of genes stems in part from potential sources of both underestimation and overestimation. Underestimation would result from the absence of genes from the microarray used, poor performance of probe sets, the inability to detect some rare mRNAs, and the fact that our calculations ignored all 953 unannotated probe sets. An example of one type of poor probe set performance appeared to be Sstr4, which was judged absent because of significant signal on its mismatch probe set features but which we detected in OSNs by in situ hybridization. Overestimation would result primarily from errors in mRNA detection by the microarray, such as uncorrected instances of cross-hybridization, absence of a method to validate independently probe sets with low signals, errors in gene annotation, and presence of subtypes of OSNs that differentially express substantial numbers of genes. Our in situ hybridizations did not provide evidence for such distinctly different subtypes of OSNs, and the agreement of the in situ hybridization results with the microarray data begins to suggest that overestimation was relatively rare. Overall, the factors contributing to underestimation seem to outweigh overestimation, indicating that our calculations were probably conservative.

Furthermore, our calculation of 10,000 genes expressed by OSNs agrees with computational predictions of the number of genes expressed in a human cell and with projections based on the expression profiling of purified CNS neurons that examined a smaller fraction of the mouse genome (Kuznetsov, 2002; Sugino et al., 2006). Our calculation also agrees closely with a study that used a
clone-and-count method of expression profiling to estimate that a cultured cell line derived from human breast epithelium expressed 10,000 genes and that a colon adenocarcinoma cell line expressed 15,000 genes (Jongeneel et al., 2003). Our approach improves on these estimates by incorporating in situ hybridization as an independent method of confirming the expression patterns predicted by expression profiling so that statistical methods could be used to substantiate the microarray data. That our numbers agree with previous estimates from other mammalian cell types lends further support to their accuracy. In addition, that we arrived at a similar number using a cell...
type purified directly and rapidly from live mice lends credence to extending these previous estimates to cells in vivo. Do these calculations, based on measures of mRNA, mean that 10,000 different proteins are found in most cells? Probably so, because measures of mRNA and protein expression in yeast reveal a significant overall correlation not only in the presence of both but also in amount (Grunenfelder and Winzeler, 2002; Ideker et al., 2001). Overall, these data represent the best evidence yet that a single type of mammalian cell expresses such a large fraction of the genome.

Biological processes enriched in OSNs

A unique contribution of expression profiling data is the ability to detect groups of functionally related gene products, thereby providing strong evidence of functions performed by the source cell or tissue. Recent development of methods that apply statistical tests to identify over- and underrepresented processes have greatly improved confidence in the reliability of these analyses (see, e.g., Hosack et al., 2003). In this project, the expression profiles of the GFP+ and GFP− samples further confirm and expand our previous evidence that chromatin remodeling and gene silencing are highly active in OSNs and their progenitors (Shetty et al., 2005). This may reflect the molecular events that underlie the cellular dynamics necessary for continuous neurogenesis. The expression patterns of Polycomb complex genes across the olfactory epithelium in our data suggest a correlation between the degree of cellular differentiation and expression of Polycomb initiation complex genes (PRC2) vs. Polycomb maintenance complex genes (PRC1), which establish and maintain the silenced state of certain genes, respectively (Muller and Leutz, 2001; Raurporst, 2005). In the olfactory epithelium, components of both complexes were expressed either in OSNs or in basal cells along with OSNs. As a group, the initiation complex members tended to have a lower mean GFP+/GFP− ratio (0.9 ± 0.5) than the maintenance complex (1.5 ± 1.3; Student’s t = 1.16, 21 df; one-tailed P < 0.13), consistent with the typical developmental progression of Polycomb gene expression patterns. The unusually large number of genes expressed specifically in OSNs and the remarkable ability of each OSN to select for expression just one odorant receptor allele may also contribute to a heightened need for chromatin remodeling (McClintock, 2000; Mombaerts, 2004). We found that OSNs and their progenitors express genes belonging to several types of chromatin remodeling processes, including Polycomb complexes, SWI/SNF complexes, NuRD complexes, and related histone deacetylases. Whether this diversity represents a coordinated effort to regulate and maintain gene expression that produces the mature OSN phenotype or independent processes that regulate expression of genes specific to certain functions is a question for further study.

Our results also confirm and extend our previous evidence that OSNs express a common set of genes involved in the formation and maintenance of cilia and flagella (Shetty et al., 2005). Because ciliogenesis is activated during the differentiation of immature OSNs into mature OSNs (Cuschieri and Bannister, 1975; Farbman, 1994; Schwarzenbacher et al., 2005), gene expression supporting this process should be enriched in the population of mature OSNs that we purified by FACS of cells from the epithelia of OMP-GFP mice. That cilia, flagella, and spermatogenesis were overrepresented processes in the GFP+ sample provides molecular evidence supporting the cellular events. The molecular evidence suggests that OSNs not only begin to express components of the cilium proper but also alter the somatodendritic compartment to support the cilia, including expression of genes responsible for the processing and trafficking of proteins that reside in cilia or flagella. Components of cilia or flagella expressed by OSNs include Spa17, Croc4, Gas8, Fsp1, and Tep11. OSNs also expressed genes encoding proteins critical for basal bodies and centrioles that support cilia and flagella. These include Bbs2, Bbs4, Bbs5, Bbs7, and Odf2 (Donkor et al., 2004). Trafficking and chaperone proteins that support cilia and flagella were expressed in OSNs, including Clgn, Phtf1, Cct6b, and Ift172 (Bourke et al., 2002; Oyhenart et al., 2003). The transcription factors that control this change in phenotype are not yet defined, but combining arguments by analogy to spermatogenesis or expression in testis with our evidence of which transcription factors are enriched in OSNs suggests that good candidates are Rfx3, Rfx4, Zmynd10, Foxj1, and Angptl6. The functions of these signals in the olfactory system are a mystery. Their functions in other systems provide clues, however. For example, Angptl6 and Ecgf1 are potent angiogenic factors, and this may explain our observation that Sox18, a transcription factor found primarily in vascular epithelial cells during angiogenesis, is abundantly expressed in blood vessels underlying the olfactory epithelium even in mice at age 3 weeks (R.S. Shetty, S.C. Bose, and T.S. McClintock, unpublished data). Btc and Fgf9 have proliferative effects and are often active on glial cells (Dunbar and Goddard, 2000; Oike et al., 2003). They and several other growth factors we detected in OSNs are known to be expressed in neurons, suggesting that OSNs use them to signal to cell types common to neural tissue, such as glia or postsynaptic neurons (Cinaroglu et al., 2005; Peretto et al., 2002; Zheng et al., 2004). That OSNs express growth factors that target neighboring cells already has precedents (Getchell et al., 2002b). In contrast, the known intracellular actions of two of the growth factors in other cells, Hdgf and Fgf12, suggest that they may have autocrine-like functions in OSNs (Kishima et al., 2002; Liu et al., 2001).

OSNs appear to respond to a surprisingly large number of signals that activate GPCRs, given that we detected mRNAs for at least 30 nonchemosensory GPCRs in the GFP+ sample. This is far beyond the previous evidence that OSNs respond to dopamine and arginine-vasopressin (Koster et al., 1999; Levasseur et al., 2004). OSNs are glutamatergic (Berkowicz et al., 1994), consistent with our finding that OSNs contain mGlur4 mRNA, whose encoded protein is typically found at glutamatergic presynaptic terminals (Schopp, 2001). We found Sstr4 mRNA in OSNs, suggesting that OSNs may be a target of the somatostatin-expressing subtype of periglomerular cells, a bulbar interneuron whose dendrites receive input from OSN axons (Gutierrez-Mecinas et al., 2005; Takami et al., 1990). OSNs express Htr1b, a serotonin receptor, and our...
data suggest they also express the closely related Htr1a, plus a muscarinic acetylcholine receptor, Chrnl. Centrifugal serotoninergic and cholinergic inputs to the olfactory bulb are potential sources of agonists for these receptors (Bouvet et al., 1988; Gomez et al., 2005; Wilson et al., 2004). OSNs express at least one of the frizzled receptors, Fzd3, and probably also express Fzd10. We also confirmed that OSNs express Ryk, an alternative Wnt receptor, further indicating that OSNs are sensitive to Wnt ligands. Indeed, we detected expression of three Wnt genes in the olfactory epithelium, Wnt11, Wnt4, and Wnt5a. The GBP+/GBP− ratio data predict that Wnt11 is expressed in OSNs and that Wnt 4 is expressed in cells other than mature OSNs. Other GPCRs that were predicted to be expressed in OSNs by our data include several linked to cell adhesion processes that mediate the behavior of neurites and axon terminals, such as the Celsrs, and the latrophilin receptors Lphn1–3. These proteins participate in the formation of axon tracts, synapses, and dendritic arbors in other parts of the nervous system (Kreienkamp et al., 2002; Tissir et al., 2002; Ye and Jan, 2005).

We also detected numerous other cell adhesion molecules in the GBP+ sample. Given the interest in discovering potential identity markers that would complement the specific identities provided by the exclusive expression of odorant receptor alleles, it is intriguing that our data predict expression in OSNs of three types of cell adhesion genes that either belong to large gene families or are capable of extensive alternative splicing to generate a great diversity of products: Nrxn1, Dscam, and protocadherins (Frank and Kemler, 2002; Hummel et al., 2003; Ullrich et al., 1995). We have not yet attempted to determine whether any of these genes and their alternatively spliced products are differentially abundant across the population of OSNs, a necessary condition if they perform addressing functions for OSNs. However, ligands for Nrxn1 are known to be expressed by postsynaptic targets in the olfactory bulb (Clariss et al., 2002). Also critical for OSN axon pathfinding are receptors for semaphorins, neuropilins, and plexins (Cloutier et al., 2004; Schwarting et al., 2004; Taniguchi et al., 2003; Walz et al., 2002). We detected neuropilins and plexins in the GBP+ sample, and several had GBP+/GBP− ratios >1.3, predicting their expression in OSNs. We suspect that the neuropilins and plexins with lower ratios are also expressed in the OSNs, probably most abundantly in immature OSNs, because Nrp2, which had a ratio of 0.7, is expressed in OSNs (Walz et al., 2002).

Another source of signals to and from OSNs may be tight junctions. The dendritic knobs of OSNs make tight junctions with neighboring sustentacular cells (Farbman, 1992). The detection of components of tight junctions and their downstream signaling molecules, such as cadherins and catenins, in the GBP+ sample supports these morphological data. The consequences of catenin signaling for OSNs are entirely unknown.

Transcription factors expressed by OSNs

The number of transcriptional regulators that our data predicted OSNs express, at least 268, is large but is consistent with a previous estimate from a cultured human cell line (Jongeneel et al., 2003). To make sense of the wealth of expression of this type of gene in OSNs, it helps to focus on challenges faced by OSNs. Transcriptional regulators expressed in OSNs should fall into two broad categories that roughly correspond to the mature and immature phenotypes that coexist in the olfactory epithelium. Those mRNAs encoding transcriptional regulators involved in differentiation should show preferential expression in the immature OSN layer in our in situ hybridization data. The mRNAs showing this pattern of expression were Mef2b, Mef2a, Max1, Tle1, Tle3, and Eya2. All of these are known to be associated with developmental events in other tissues. Homeodomain transcription factors are probably important for the final differentiation and function of OSNs (Hirotani and Mombaerts, 2004; Vassalli et al., 2002). We detected several in the GBP+ sample, including Lhx2, Phtf1, Phtf2, Pknox1, Pknox2, En2, Pbx2, Pbx3, Hod, Zhh1, Zhh2, Zhh3, and Unecn4.1. However, this project was not optimally designed to identify mRNAs involved in OSN development and differentiation. Probing gene expression during active replacement of OSNs was more effective at detecting transcription factors expressed primarily by immature OSNs (Shetty et al., 2005).

Transcription factor mRNAs more abundant in mature OSNs are more likely to be associated with maturation and homeostasis of differentiated cells. This was true of Elk4, Etv3, Cebh311, Atf4, Atf6, Phx1, Phx2, En2, Tead1, Unecn4.1, Nfatc1, Bf3x3, Bf3x4, Nfe2l1, Nfe2l2, Eomes, and Zmynd10 (Araki et al., 2004; Horsley and Pavlath, 2002; Janknecht and Hunter, 1997; Qiu et al., 2004; Sawka-Verhelle et al., 2004; You et al., 2004). Many of these mRNAs are known to have antiproliferative activity, to be involved in stress responses, or to support the production of cilia or flagella. We detected several transcription factors known to repress the cell cycle: Eto2, Eto3, Mnt, E2f6, E2f5, Mad, Bdr7, and Ing1. More than any other mammalian neuron, OSNs are exposed to damage from chemicals and pathogens. Stress response transcription factors were numerous among those that we predict are expressed in OSNs: Atf4, Atf6, Ahr, Arnt, Kep1, Nfe2l2, Nfe2l1, Mlll3, Bcl6, Maz, Nfat5, Nfat1, Usf2, and Ches1. OSNs must also produce and support cilia, consistent with the detection in the GBP+ sample of several transcription factors associated with ciliogenesis or spermatogenesis, as described above. OSNs specifically express a large number of genes, such as components of the olfactory transduction pathway and the odorant receptors, so transcription factors that contribute to this specificity should have appeared in our data. Although all such genes might not have been identified, we certainly detected the members of the Ebf family, which are critical for OSN-specific expression of several genes (Wang et al., 2004). Transcription factors activated by neuronal activity and calcium flux are also likely to be important for OSN survival and homeostasis. We detected several transcription factors of this type, including Tle3, Nfatc1, Nfatc3, and Nfat5.

A gene expression resource for the olfactory epithelium

We have found the expression profiles of the GBP+ and GBP− samples to be extremely useful in thinking about the function of cells in the olfactory epithelium, especially of OSNs. Although we have identified several biological processes enriched in OSNs and a number of individually interesting genes, we have only scratched the surface of the wealth of information present in these data. To make this resource freely available in the most flexible format so
that others can also mine these data, we provide them in an Excel file (Supplementary Table 4). This file contains processed data that correspond directly with the interpretations discussed herein and should be used solely within the limitations discussed above. A GFP+/GFP− ratio > 1.3 accurately predicts expression in OSNs; the higher the ratio, the more likely that the mRNA is restricted to mature OSNs. Ratios of 1.2–0.5 are less predictive, so, although most members of this category are expressed in OSNs, they often can be found in other cell types in the olfactory epithelium. Ratios <0.4 begin to predict expression that is restricted to immature OSNs and nonneural cell types in the olfactory epithelium.

These data should be broadly useful in the study of OSN function. Most interesting in the long term are the thousands of OSN-expressed genes whose functions are either completely unknown or only crudely predicted from sequence similarity. As difficult as it may be, these genes must be investigated. They provide immediate avenues toward experiments with real hope of novel discoveries. These data not only identify these mRNAs but also provide measures of expression levels and enrichment in OSNs to help guide selection of those genes most likely to play significant roles in OSN function.

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