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Mammalian odorant receptors: functional evolution and variation

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Abstract

In mammals, the perception of smell starts with the activation of odorant receptors (ORs) by volatile molecules in the environment. The mammalian OR repertoire has been subject to rapid evolution, and is highly diverse within the human population. Recent advances in the functional expression and ligand identification of ORs allow for functional analysis of OR evolution, and reveal that changes in OR protein sequences translate into high degrees of functional variations. Moreover, in several cases the functional variation of a single OR affects the perception of its cognate odor ligand, providing clues as to how an odor is coded at the receptor level.

Odorant receptors (ORs), a class of 7-transmembrane G protein-coupled receptors (GPCRs) expressed in the olfactory sensory neurons (OSNs) that line the main olfactory epithelium in nasal cavity, detect volatile chemicals in the environment [1]. Odor recognition is integrative, in that one odor can activate multiple ORs, and one OR can respond to multiple odors, which is often referred to as the combinatorial coding of odors [2]. Therefore, it is fundamental to know which and how many ORs are activated by a given odor, and how each OR contributes to odor perception.

Mammalian genomes typically encode large numbers of ORs (Table 1), with approximately 400 intact ORs in human, more than 1000 in mouse and close to 2000 in African elephant [3–5]. An exception is found in cetaceans (whales and dolphins), which only have small numbers of intact ORs [6]. The ORs are undergoing rapid evolutionary changes including extensive gene gains and losses, as seen by a high percentage of OR pseudogenes in mammalian genomes (Fig 1 A, B) [7]. ORs also exhibit high genetic variability within the

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human population (Fig 1 C) [8]. Thus it is intriguing to ask how the functions of ORs change during evolution, and what the consequences of such functional changes are.

To address these questions, an unbiased assay for quantifying the functions of ORs, specifically their ability to respond to cognate ligands, is required. However, the majority of ORs are highly resistant to heterologous expression when expressed alone, greatly limiting our ability to compare variants of ORs at the functional level. For example in HEK293T cells, most ORs fail to exit the endoplasmic reticulum (ER) and are not functionally expressed at the cell surface [9]. The identification of receptor transporting proteins (RTP1, RTP2) that facilitate OR trafficking [10], and subsequent identification of a shorter and more potent form of RTP1, RTP1S [11], has greatly enhanced the ability to functionally express ORs *in vitro*. Later studies found that RTP1S acts as a chaperone that facilitates OR trafficking at several steps including exiting from the ER and trafficking from the Golgi to the cell surface [12]. RTP1S may also serve as a co-receptor by forming complexes with ORs on the cell surface, as disruption of the association between RTP1S and ORs on the cell surface severely impairs OR function [12]. In addition, Ric-8B, a guanine nucleotide exchange factor [13], amplifies the Golf signaling cascade downstream of OR activation [14]. Furthermore, another GPCR, the type 3 muscarinic acetylcholine receptor (M3-R), potentiates the response of many ORs without altering their ligand selectivity [15]. A heterologous system based on these cofactors for OR expression aided the deorphanization of ~40 human ORs and ~95 mouse ORs [16,17], providing a platform for the functional analysis of variations in mammalian OR repertoires.

Central to understanding the functional evolution of ORs is functional comparison within orthologous ORs (ORs evolved from a common ancestral OR by speciation) as well as within paralogous ORs (related ORs by duplication within a genome) (Fig 1 B). It is usually assumed that orthologs perform equivalent functions, while paralogs may be more diverged in function [18], but the extent to which this assertion holds for ORs needs to be tested. For instance, the human OR OR1A1 and its mouse ortholog Olfr43 show similar responses to (S)-(-)-citronellol [19], supporting the notion that orthologous ORs are equivalent in function. However, the mouse and rat I7 orthologs show different preferences for heptanal and octanal, as a result of a single amino acid change [20]. In addition, in a study involving orthologs of Olfr154 (OR912-93) across multiple species, the ligand selectivity of orthologs was found to be similar but not the same: the Olfr154 orthologs of mouse, pig and four primates all responded to both 2- and 3-heptanone, the squirrel-monkey ortholog only responded to 3-heptanone, and the human and orangutan orthologs were not functional [21]. When quantitative characteristics of OR responses were taken into account, primate orthologs of the human OR OR7D4 exhibited extremely diverse potency and efficacy to two steroid ligands, androstenedione and androstadienone [22]. Finally, a large scale analysis of 18 OR orthologs in the human, chimpanzee and/or rhesus macaque and of 17 in mouse and rat against a panel of 42 ORs revealed that a striking ~90% of orthologous ORs showed functional variances affecting receptor potency and/or efficacy (Fig 2 A, B). When only considering binary responses, orthologous ORs respond to a common ligand 83% of the time, while paralogs respond to a common ligand only 33% of the time [23]. These studies suggest that the ligand selectivity of OR orthologs are more conserved than paralogs, while

the response ranges among orthologs can dramatically differ. In the future, it will be important to evaluate how the functional differences in OR orthologs and paralogs contribute to the adaptation of different species.

The OR repertoire is highly variable across the human population (Fig 1 C), encompassing large numbers of single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) [24–27]. A human OR has a median of five different alleles with an allele frequency greater than 1% in the 1000 Genomes Project data [17]. Recently, a functional analysis of variants of 27 human ORs in the heterologous expression system revealed that 63% of these ORs have variations that alter *in vitro* functions, and two individuals have an average of over 30% functional differences in their OR alleles [17]. Among the variants that alter OR protein sequences, 7.9% lead to nonsense mutations that would disrupt ORs, 6.8% are hypofunctional (lower sensitivity and dynamic range) and 11% are hyperfunctional (higher sensitivity and dynamic range) as compared to reference alleles (Fig 2 C) [17]. In this study, only SNPs in the open reading frames of ORs were considered. Additional functional differences could result from variations in the regulatory regions that control OR expression, as well as CNVs that cause gains and losses of certain ORs. Recent transcriptome analysis is starting to reveal differences in OR expression levels among individuals [28]. CNVs are also often observed [29], with reported CNVs for ~33% of the human ORs, and an average of 8 CNVs of OR loci per individual [25].

Humans exhibit remarkable inter-individual variations in odor perception [30]. While the extent to which these are explained by OR variations remains unclear [31], functional variations of single ORs have been shown to confer differences in perceived intensity and/or pleasantness of their ligands, suggesting that relatively small numbers of ORs might play a dominant role in the perception of certain odors (Table 2). The first evidence linking genotypes of an OR to differences in odor perception through responses of OR variants *in vitro* came from the human OR OR7D4 [32,33]. Two linked missense variants of OR7D4 severely impaired the *in vitro* response of the receptor to androstenone and androstadienone. Subjects with these variants were less sensitive to these two steroid odors and more likely to rate them as less unpleasant. The OR7D4 genotype explained 39% and 19% of the variance in intensity rating and perceived pleasantness of these odors, respectively [32]. As androstenone occurs in pork derived from male pig, the OR7D4 genotype is also shown to affect food preference, with subjects carrying the more sensitive OR7D4 variant tending to rate the androstenone-containing meat as less favorable [34]. The OR7D4 genotype is also correlated with the rating of partner odors in heterosexual partners, which in turn associates with scores of anxiety and depression [35]. A variant of an OR pseudogene OR11H7P leads to a functional receptor that responds to isovaleric acid. Subjects with this functional receptor are on average more sensitive to isovaleric acid. The genotype of OR11H7P explains 8% of the variance in isovaleric acid detection threshold [36]. OR2J3 responds to cis-3-hexen-1-ol, a grass-like odor, and two missense variants of OR2J3 explain 26% of the variance of detection threshold for cis-3-hexen-1-ol [37]. OR10G4 genotype explains over 15% variation in perceived intensity and 10% variation in perceived pleasantness of one of its *in vitro* ligand, guaiacol [17]. Taken together, for the aforementioned four ORs, variance in odor perception is partially explained by changes in the OR function. The moderate

proportions perhaps reflect the combinatorial coding scheme of odors in that the perception of odors usually requires activation of several ORs, or differences in OR expression levels across individuals [28], in addition to possible non-genetic factors. In contrast, a SNP causing a single amino acid change in an OR that responds to β -ionone *in vitro*, OR5A1, explains 96% of variance in β -ionone perception, providing an extreme example in which the functional variance of a single OR almost completely determines the perception of its ligand [38]. Although several other ORs also responded to β -ionone *in vitro*, OR5A1 displayed a ~10 times higher sensitivity [38]. The OR5A1 genotype also causes differences in preference towards food and products that contain β -ionone [38]. In addition, variations in perception for several odors have been mapped to clusters of ORs, although the responsible ORs are yet to be determined. These odors include 2-heptanone, isobutyraldehyde, β -damascenone [39], soapy aroma of cilantro [40], and urine odors after consuming asparagus [41]. The ORs within the identified clusters are interesting candidates to test in functional assays to determine causal variants.

Small changes in OR protein sequence can cause significant differences in their functions both *in vitro* and *in vivo*. However, how specific amino acid residues determine the ligand selectivity and activity of ORs remains unclear. Molecular docking and model guided mutagenesis have been used to pin down the residues involved in ligand binding [42]. However, these efforts are currently limited by the lack of OR crystal structures, in spite of advances in the structural biology of other GPCRs [43].

The heterologous system has been useful in determining the functional differences of ORs. Studies including those of M71, M72, I7, OR-EG, MOR23, SR1 in mice, and OR7D4, OR11H7, OR5A1, OR2J3, OR10G4 in humans have shown that *in vitro* responses predict OSN activation in mice and behavior in humans [17,32,36,44,45]. However, it should be noted that the heterologous system has its limitations, and cautions should be used when interpreting the *in vitro* results especially when comparing responses of different receptors to a common ligand. For instance, the surface expression levels of ORs should be quantified to exclude the possibility that different levels of OR expression on the cell surface cause differences in response. Future study should address how *in vitro* response correlate with OSN activation in a comprehensive manner.

Although ORs are mainly expressed in the olfactory epithelium, some ORs can be detected in non-olfactory tissues. For example the mouse OR, Olfr78 (also known as MOR18-2), is expressed in the kidney tissues, mediating renin release when activated by its ligand propionic acid [46]. The human homolog of Olfr78, OR51E2, and its paralog OR51E1, are highly expressed in prostate cancer cells and were originally characterized as prostate cancer markers [47,48]. Activation of OR51E1 inhibits cancer cell proliferation [49], but promotes invasiveness and metastasis [50]. Another example is the human OR1D2 (also known as OR17-4), which is found in the testis. A ligand of OR1D2 induces a calcium response in sperm cells and mediates their chemotaxis [51]. Recent transcriptome analysis of human tissues detected the transcripts of more than 100 ORs in non-olfactory tissues [52]. While the functional significance of the majority of ectopically expressed ORs needs further investigation, there is evidence that the ectopic expression of ORs is likely a regulated process, and may be functional in some aspects. For instance, the ORs expressed in a non-

olfactory tissue in human also tend to be expressed in the same tissue in chimpanzee [53]. In addition, toothed whales lack olfactory structures [54], but their genomes do encode a small number of intact ORs, implying possible non-olfactory functions. Hence, although mainly speculation at this point, it is possible that variants of ectopically expressed ORs can cause effects outside the olfactory system. For ORs that have additional functions outside the olfactory epithelium, we must consider different evolutionary constraints and selection pressures based on the non-olfactory functions of each OR.

Future directions

While rapid and dynamic functional evolution of the OR repertoire is evident across different species and within the human population, how these changes relate to the fitness and adaptation is unclear. Despite of years of effort, ligand information for the vast majority of mammalian ORs is still lacking. Comprehensive identification of receptor repertoires for representative odorants is desirable to deepen our understanding of odor coding. In addition, current evaluation of OR functions heavily relies on heterologous systems, and an *in vivo* system that allows for functional analysis of ORs is thus desired in order for results to be compared [55]. Furthermore, structural information of ORs is crucial for understanding how protein sequence variations in ORs link to their functional differences.

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Highlights

- Mammalian ORs undergo rapid evolution at the primary sequence and functional levels
- Functional differences are more frequently observed among OR paralogs than orthologs
- OR orthologs show conserved ligand selectivity, yet can be different in their sensitivity and/or efficacy
- Human OR repertoire displays high degrees of polymorphisms which translate into functional changes
- Functional variation of ORs partly explains differences in odor perception in humans

Figure 1. Functional evolution and variation of ORs

(A) Expansion of the OR repertoire. During evolution, an ancestral OR gene is duplicated. The gained copy of this OR can become a pseudogene (pseudogenization), or obtain new functions such as responsiveness to a different odor (neofunctionalization). In addition, the two copies of the ancestral OR can each maintain a subset of the original function, each responding to subsets of the ligands of the ancestral OR (subfunctionalization).

(B) OR orthologs and paralogs. Orthologs are defined as genes related via a speciation event (for example, human OR 1 and chimpanzee OR 1). Paralogs are defined as genes related via a gene duplication event (for example, mouse OR 1 and mouse OR 2).

(C) ORs exhibit high degrees of variation within human populations. A given OR can have several variants with altered protein sequences, and the functions of these variants are sometimes also altered.

Figure 2. Summary of functional variations among ORs

(A) Change of *in vitro* responses of 17 human ORs with respect to chimpanzee orthologs. (B) Change of *in vitro* responses of 17 mouse ORs with respect to rat orthologs. (C) Functional variations among 27 human ORs of 1092 subjects in the 1000 Genomes Project. The functional variations are classified as hypofunctional (one OR has a lower potency (EC50) and efficacy (dynamic range)), hyperfunctional (one OR has a higher potency and efficacy), indistinguishable, or unclassified (the ORs have significantly different *in vitro* responses but the potency and efficacy do not change concordantly) when comparing the

responses of two variants to a shared cognate ligand. Each pie chart refers to the first species in the comparison: (A) human OR is ____ to chimpanzee; (B) mouse OR is ____ to rat; (C) 1000 Genomes OR is ____ to the reference allele.

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Table 1

Numbers of OR genes in the genomes of several mammal species as reported in [5]. The truncated OR was defined as a partial, intact sequence located at a contig end, and may be either a functional OR or a pseudogene [5].

Species	Intact ORs	OR pseudogenes	Truncated ORs
Human	396	425	0
Chimpanzee	380	414	19
Mouse	1130	236	0
Rat	1207	508	52
Dog	811	278	11
Elephant	1948	2230	89

Table 2

Genetic variations of ORs associated with changes in odor perception. In the first 5 cases (ordered by publication date), the variation within a single OR causes changes in OR function *in vitro*, and explains variation in odor detection. In the other 5 cases (ordered by publication date), variations in odor detection map to OR clusters, which are labeled by the peak SNPs of association (SNPs most-significantly associated with the phenotypes).

	OR/peak SNP within OR cluster	Odor ligands	OR variant	Change of OR function in vitro	Reported phenotype associated with variant	Percentage of variance explained	References
ORs	OR7D4	androstenone, androstadienone	R88W,T133M	diminished response to ligands	higher detection threshold	39% intensity rating	32–35
	OR11H7P	isovaleric acid	pseudogene to intact		rate ligands as less intense and less unpleasant	19% perceived pleasantness	
	OR2J3	cis-3-hexen-1-ol	T113A,R226Q	obtained response to the ligand	rate the androstenone-containing meat as less unpleasant		36
	OR5A1	β-ionone	N183D	diminished response to the ligand	changes in rating of partner odors		37
				increased sensitivity and response	lower detection threshold	8%	38
OR clusters	OR10G4	guaiacol	A9V,M134V,V195E,R235G,K295Q	amplitude to the ligand	less likely to prefer food containing the ligand and more likely to prefer non-food products containing the ligand		
			T62I,Y101C,M134V,G146S,P181S,V195E,R235G,K295Q	diminished response to the ligand	rate the ligand as less intense and more pleasant	10% perceived pleasantness	17
OR clusters	rs4481887, chromosome 1	urine odor after consumption of asparagus	NA	NA	ability to detect the odor	NA	41
	rs72921001, chromosome 11	soapy aroma of cilantro	NA	NA	variation in perception and preference for cilantro	NA	40
	rs13424612, chromosome 2	isobutylaldehyde	NA	NA	variation in detection threshold	NA	39
	rs9809531, chromosome 3	2-heptanone	NA	NA	variation in detection threshold	NA	39
	rs2220004, chromosome 11	β-damascenone	NA	NA	variation in detection threshold	NA	39