

# Odorant receptor gene choice is reset by nuclear transfer from mouse olfactory sensory neurons

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**Of the ~1,000 odorant receptor (OR) genes in the mouse genome, an olfactory sensory neuron (OSN) is thought to express one gene, from one allele. This is reminiscent of immunoglobulin and T-cell receptor genes, which undergo DNA rearrangements in lymphocytes. Here, we test the hypothesis that OR gene choice is controlled by DNA rearrangements in OSNs. Using permanent genetic marking, we show that the choice by an OSN to express an allele of the OR gene *M71* is irreversible. Using *M71*-expressing OSNs as donors for nuclear transfer, we generate blastocysts, embryonic stem (ntES) cell lines and clonal mice. DNA analysis of these cell lines, whose genome is clonally derived from an *M71*-expressing OSN, does not reveal DNA rearrangements or sequence alterations at the *M71* locus. OSNs that differentiate from ntES cells after injection into blastocysts are not restricted to expression of *M71* but can express other OR genes. Thus, *M71* gene choice is irreversible but is reset upon nuclear transfer, and is not accompanied by genomic alterations.**

The discovery of OR genes<sup>1</sup> gave rise to the idea that the choice of expression of one allele<sup>2–4</sup> of one gene<sup>5</sup> out of a thousand<sup>6–9</sup> might involve DNA rearrangements, as in immunoglobulin and T-cell receptor genes<sup>10,11</sup>. A crucial distinction between these types of gene is that the complete coding region of OR genes is already present, preassembled, in the mouse germ line, whereas immunoglobulin and T-cell receptor genes are assembled from gene segments in lymphocyte nuclei by DNA rearrangements. The lack of OSN cell lines expressing an OR gene has precluded a direct search for genomic alterations. Single-cell genomic analysis is technically challenging and offers poor resolution.

Cloning by nuclear transfer has been successful in mouse for a few cell types<sup>12–16</sup>. Nuclear transfer preserves the DNA rearrangements of immunoglobulin and T-cell receptor loci that occur in the nuclei of B and T lymphocytes<sup>15</sup>, and has been proposed as a means of testing the hypothesis of DNA rearrangements of OR genes in OSNs<sup>7,15</sup>. This method allows the clonal propagation of a nuclear genome as an alternative to establishing a cell line from an OSN. However, as mature OSNs are postmitotic, it is not known whether their nuclei can be reprogrammed to re-enter the cell cycle and instruct the embryonic development of a cloned mouse. Moreover, mature OSNs cannot be obtained as a homogeneous cell type from olfactory epithelium, and OSNs expressing a particular OR gene form an extremely small population of cells within the epithelium. A permanent genetic marker is required to prove that the cloned organisms were derived from a mature OSN. As it is not known whether all mature OSNs express an OR gene, marking should ideally be specific for OSNs that express a predefined OR gene.

Here, we describe three sets of experiments with mature OSNs, permanently marked mature OSNs, and permanently marked OSNs that express the *M71* OR gene. The *M71* promoter has been characterized previously in transgenic mice<sup>17</sup>. The permanent marker strategy is based on Cre-mediated activation of a ubiquitously and constitutively expressed green fluorescent protein (GFP), allowing us to identify with certainty the origin of the donor nuclei that supported the establishment of an ntES cell line. From 1,007 successfully reconstructed oocytes, we establish 16 ntES cell lines. We show that OR gene choice is irreversible within an individual

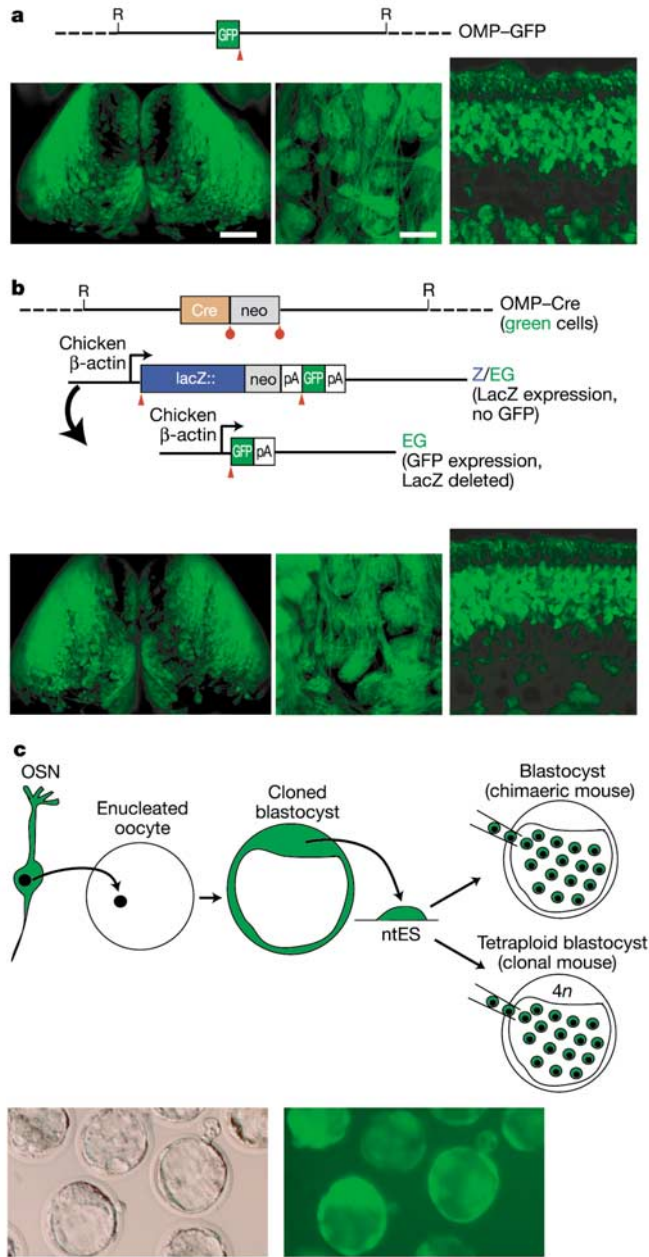
OSN but is reset by nuclear transfer, and does not involve genomic alterations. Similar conclusions are reached in ref. 18.

## Mature OSNs as nucleus donors

Mature OSNs can be discerned by specific and high-level expression of the olfactory marker protein (OMP), a protein of unknown function. In OMP–GFP mice (Fig. 1a), in which the OMP-coding region is replaced by that of the GFP, mature OSNs can be identified by their intense green fluorescence<sup>19</sup>. In our cell dissociation protocol (see Methods), ~20% of cells from OMP–GFP mice are green fluorescent. We used 411 green-fluorescent cells of OMP–GFP mice successfully as nucleus donors to establish a first set of six embryonic stem (ES) cell lines, which are conventionally referred to as ntES cell lines<sup>14,20</sup> to reflect the step of nuclear transfer (Table 1). This first set of experiments supports the feasibility of nuclear transfer with mature OSNs, but there is no proof that the nuclei of mature OSNs were the source of these ntES cell lines.

## Permanently marked OSNs as nucleus donors

To mark the nuclei of mature OSNs permanently, we generated a novel strain of mice by gene targeting, OMP–Cre, in which the OMP-coding region<sup>21</sup> is replaced by that of the site-specific Cre recombinase (Fig. 1b). Crossing these mice with Z/EG transgenic reporter mice<sup>22</sup> results in Cre-mediated excision of the lacZ-coding sequence, which allows GFP to be expressed from the chicken  $\beta$ -actin promoter. This promoter is ubiquitously and constitutively active in mouse, including in OSNs, such that an OMP–Cre  $\times$  Z/EG mouse exhibits a pattern of green fluorescence that is indistinguishable from that of an OMP–GFP mouse. OMP-expressing cells are now permanently marked: their nuclei continue to express GFP protein upon nuclear transfer, regardless of whether the *OMP* gene is still expressed. This design, developed for lineage tracing, provides retrospective proof that the nucleus came from a cell that was expressing, or had expressed, the *OMP* gene at the time of observation. From 440 oocytes successfully reconstructed with the nuclei of green-fluorescent cells of OMP–Cre  $\times$  Z/EG mice, we cloned green-fluorescent blastocysts (Fig. 1c), and established a second set of seven ntES cell lines (termed OCZ) that are all green fluorescent (Table 1). We generated chimaeric mice by injecting these cells



**Figure 1** Nuclear transfer with olfactory sensory neurons. **a**, *OMP-GFP* targeted mutation<sup>19</sup>. (Red triangles in **a** and **b** are *loxP* sites, recognition sites for Cre recombinase; R represents *EcoRI*) Bottom left, wholemount olfactory bulbs. Green-fluorescent glomeruli receive OSN axons. Bottom middle, close-up of bulb showing glomeruli. Bottom right, section through the olfactory epithelium. Mature OSNs are green fluorescent. Scale bars: left, 500  $\mu$ m; right, 100  $\mu$ m. **b**, Permanent marking. Top, *OMP-Cre* targeted mutation (red dots are *FRT* sites), the *Z/EG* transgene, and the transgene after Cre-mediated site-specific recombination (GFP, instead of lacZ, is now expressed). Bottom, same views as in **a**, showing that the *OMP-Cre*  $\times$  *Z/EG* mouse is phenotypically identical to the *OMP-GFP* mouse. **c**, Experimental design. Green-fluorescent OSNs from a *OMP-Cre*  $\times$  *Z/EG* mouse serve as nucleus donors. Reconstructed oocytes develop *in vitro* to blastocysts, from which ntES cell lines are established. Cells are then injected into diploid or tetraploid blastocysts. ‘Clonal’ mouse denotes the possibility that a small fraction of tetraploid cells contributes to the embryo proper<sup>27</sup>. Bottom left, Bright-field image of blastocysts after oocyte reconstruction with green-fluorescent OSNs from a *OMP-Cre*  $\times$  *Z/EG* mouse. Bottom right, the same blastocysts under fluorescent illumination.

into normal, diploid blastocysts. Two-colour fluorescent *in situ* hybridization (Fig. 2a–c) with probes for *OMP* and *GFP* demonstrates that these ntES cells can give rise to mature, *OMP*-expressing OSNs. Probes for a randomly selected sample of the OR repertoire, *MOR28* (ref. 23) (also known as *MOR244-1*, ref. 6) and *MOR251-4* (ref. 6), co-hybridize in some cells with a probe for *GFP* (Fig. 2d–i). Green-fluorescent OSN axons innervate multiple glomeruli, suggesting that these OSNs express a variety of ORs (Fig. 2j–l).

**M71 gene choice is irreversible**

Next, we designed a system to generate permanently marked ntES cell lines with nuclei of OSNs that express a predefined and well-characterized OR gene, *M71* (refs 17, 19, 24, 25). We have shown that mini-genes can reproduce the expression features of *M71* in transgenic mice, and have narrowed the regulatory region down to a few hundred nucleotides upstream of the transcription start site (ref. 17, and our unpublished observations). In *M71-IRES-tauGFP* mice<sup>25</sup>, OSNs expressing the targeted *M71* allele are green fluorescent as a result of co-translational regulation by the internal ribosome entry site (Fig. 3a). We generated a novel mouse strain with a red fluorescent marker based on *dsRed*<sup>26</sup>, the *M71-IRES-tauRFP<sub>2</sub>* mice (Fig. 3a). In *M71-IRES-tauRFP<sub>2</sub>*  $\times$  *M71-IRES-tauGFP* mice, which have differentially tagged *M71* alleles, red-fluorescent cells and green-fluorescent cells can be seen in equal numbers in the dorsal epithelium (Fig. 3a). No cell is yellow (from the overlay of red and green fluorescence), illustrating that expression is strictly monoallelic at the time of observation. However, an OSN may switch high-level expression among OR genes during differentiation or throughout its lifetime, while expressing one allele of one OR gene at any moment. If the hypothetical DNA rearrangements would be reversed by this switching (for instance, if induced by a transposon that jumps between promoters, or by gene conversion into an expression cassette), the permanent marker strategy with Cre recombinase is flawed, because GFP expression would not accurately report the OR that is expressed at the time of observation.

To exclude switching for the *M71* gene, we generated a novel strain of mice with an *M71-IRES-Cre* targeted mutation (Fig. 3b), in which Cre recombinase is expressed along with *M71*. By crossing, we obtained mice with two targeted mutations and one transgene: *M71-IRES-tauRFP<sub>2</sub>*, *M71-IRES-Cre* and *Z/EG*. Confocal imaging reveals a pattern of red-fluorescent cells and green-fluorescent cells in the epithelium (Fig. 3b) that is identical to the *M71-IRES-tauRFP<sub>2</sub>*  $\times$  *M71-IRES-tauGFP* cross (Fig. 3a). Likewise, the red-fluorescent and green-fluorescent axons co-converge into the same glomeruli in the olfactory bulb (Fig. 3c–e), indicating that both populations are biologically indistinguishable. Thus, at the time of observation the green-fluorescent cells were expressing the *M71-IRES-Cre* allele instead of having switched expression to another OR gene, and they did not express *M71* transiently from both alleles in their past.

**Permanently marked M71 OSNs as nucleus donors**

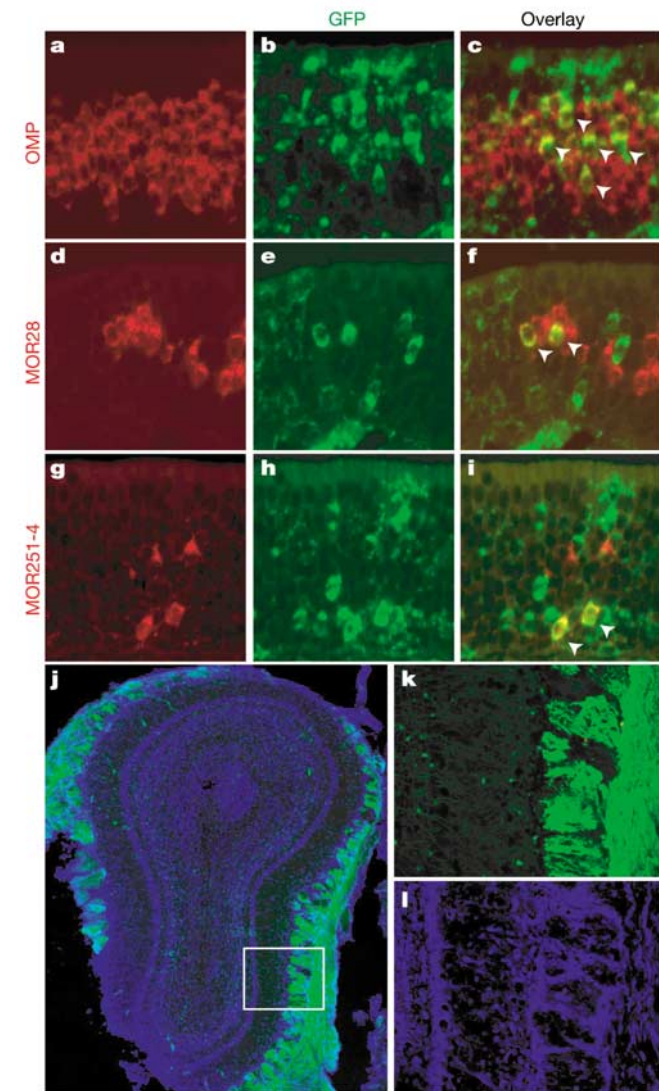
The irreversibility of *M71* gene choice is essential for interpretation of the third series of nuclear transfer experiments, with green-fluorescent cells of *M71-IRES-Cre*  $\times$  *Z/EG* mice. From 156

Table 1 Establishment of ntES cell lines by nuclear transfer from OSNs

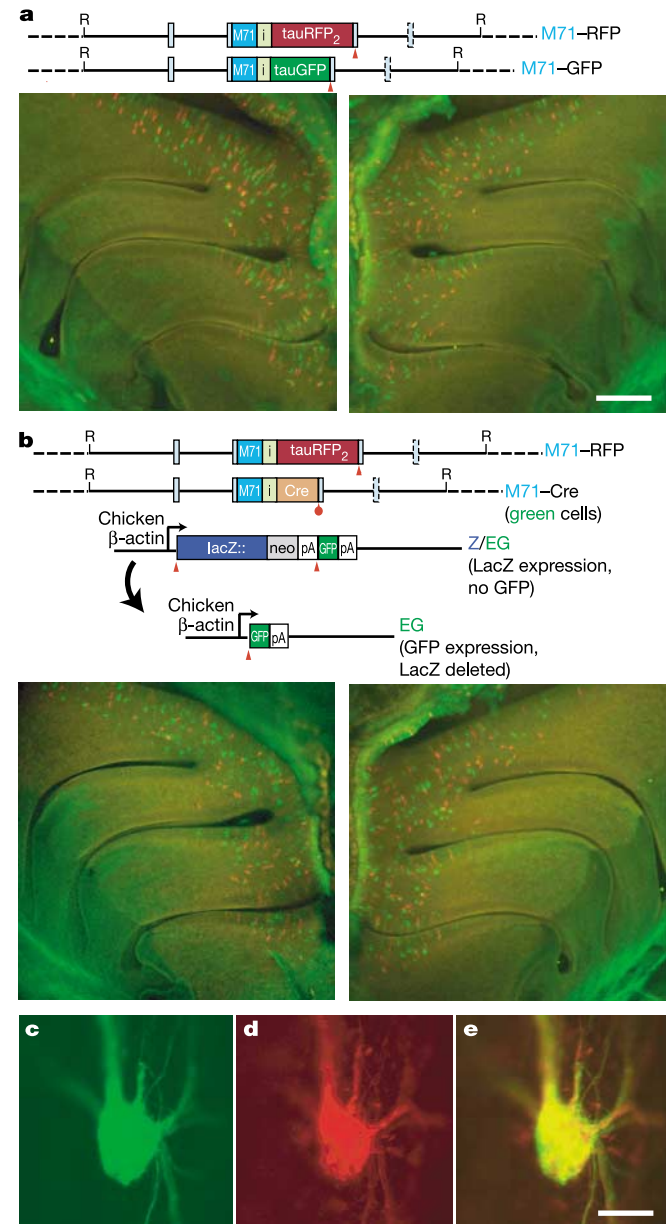
Donor strains	Reconstructed oocytes	Blastocyst development (%)	Blastocysts used for ntES cell derivation	ntES cell lines (%)
<i>OMP-GFP</i>	411	51 (12.4)	37	6 (16.2)
<i>OMP-Cre</i> $\times$ <i>Z/EG</i>	440	37 (8.4)	25	7 (28.0)
<i>M71-Cre</i> $\times$ <i>Z/EG</i>	156	17 (10.9)	12	3 (25.0)
Total	1,007	105 (10.4)	74	16 (21.6)

successfully reconstructed oocytes, we established three ntES cell lines, termed M71CZ (Fig. 4a–e). Surprisingly, only two cell lines were green fluorescent. The remaining cell line, M71CZ2 (Fig. 4e), was erroneously generated with a nucleus of a cell that was not green fluorescent: genomic DNA of M71CZ2 did not show evidence of Cre-mediated removal of the *loxP*-flanked lacZ-coding region (data not shown). Injection of green-fluorescent M71CZ cells into normal, diploid blastocysts resulted in mice with extensive somatic chimaerism (Fig. 4f, i). We also generated mice by injecting these cells into tetraploid blastocysts. As expected, the cells of these host blastocysts do not contribute to the embryo proper but only to the extra-embryonic tissues, to which, conversely, ES cells cannot contribute (Fig. 4g, h, j). (Because tetraploid cells can make

minor contributions to the embryo proper in certain cases<sup>27</sup>, the ensuing mice are not necessarily cloned—that is, derived from a single cell—but can be chimaeric; to acknowledge this uncertainty, we refer to these mice as ‘clonal’). Figure 4h shows Harvey, a 3-week-



**Figure 2** Chimaeras produced with OCZ1 ntES cells generated from permanently marked, OMP-expressing OSNs. **a–i**, Olfactory epithelium, *in situ* hybridization. Probe for *OMP* in red (**a**); probe for *GFP* in green (**b**); overlay of both (**c**). Arrowheads indicate *OMP*-positive cells derived from the injected ntES cells. Note that some *GFP*-positive cells do not express *OMP*; these are most probably supporting cells. Probe for *MOR28*, expressed in the ventral epithelium, in red (**d**); probe for *GFP* in green (**e**); overlay (**f**). Some *GFP*-positive cells express *MOR28*. Probe for *MOR251-4*, expressed in the intermediate region of the epithelium (**g**); probe for *GFP* (**h**); overlay (**i**). Some *GFP*-positive cells express *MOR251-4*. **j–l**, Olfactory bulb, fluorescence for GFP and TOTO-3, a blue nuclear dye. Green-fluorescent axons innervate multiple glomeruli (**j**). Box is magnified in **k** (intrinsic fluorescence of GFP) and **l** (TOTO-3, blue). Chimaeras are 2 months old.



**Figure 3** Expression of the *M71* OR gene is strictly monoallelic and does not switch to other OR genes. **a**, Top, *M71*-IRES-*tauRFP2* and *M71*-IRES-*tauGFP* targeted mutations. Bottom, wholemount confocal images of the left and right turbinates of an individual 4-week-old mouse with differentially tagged *M71* alleles. Cells are either red fluorescent ( $n = 224$ , 48.3%) or green fluorescent ( $n = 240$ , 51.7%) but not both, and are intermingled in the dorsal epithelium. Scale bar, 500  $\mu\text{m}$ . **b**, Top, *M71*-IRES-*tauRFP2* and *M71*-IRES-*Cre* targeted mutations, and cross with Z/EG transgenic reporter mouse. Compare with Fig. 1b. Bottom, wholemount confocal images of the left and right turbinates of an individual 4-week-old mouse heterozygous for *M71*-IRES-*tauRFP2*, heterozygous for *M71*-IRES-*Cre*, and hemizygous for Z/EG. Cells are either red fluorescent ( $n = 216$ , 49.8%) or green fluorescent ( $n = 218$ , 50.2%) but not both. The pattern is indistinguishable from **a**. **c–e**, Wholemount confocal image of glomerulus of mouse heterozygous for *M71*-IRES-*tauRFP2*, heterozygous for *M71*-IRES-*Cre*, and hemizygous for Z/EG. Green fluorescence (**c**), red fluorescence (**d**), overlay (**e**). The *M71*-IRES-*tauRFP2* and *M71*-IRES-*Cre* alleles are phenotypically identical. Scale bar in **e**, 50  $\mu\text{m}$ .

old male clonal mouse resulting from injection of M71CZ3 into a tetraploid blastocyst. The generation of chimaeric and clonal mice demonstrates that M71CZ cells do not harbour a genomic alteration that would preclude differentiation into somatic cell types.

The unlimited availability of genomic DNA from the immortal M71CZ ntES cell lines permits a search for DNA rearrangements by conventional methods. We sequenced a region of 1 kilobase in and around the *M71* promoter region that we defined previously in transgenic mice<sup>17</sup>, and found no polymorphisms between the *M71-IRES-Cre* (expressing) and wild-type (non-expressing) *M71* allele (Fig. 5a). Additionally, long-range polymerase chain reaction (PCR) between the *M71* promoter region and the *IRES* sequence showed no polymorphisms. Southern blot hybridization with a probe corresponding to the promoter and 5' nontranslated regions did not reveal a band of an altered length indicative of a genomic alteration such as gene conversion (Fig. 5a). We cannot exclude the possibility that a DNA rearrangement took place at distant sites, outside the minimal region for expression that we defined with transgenes<sup>17</sup>.

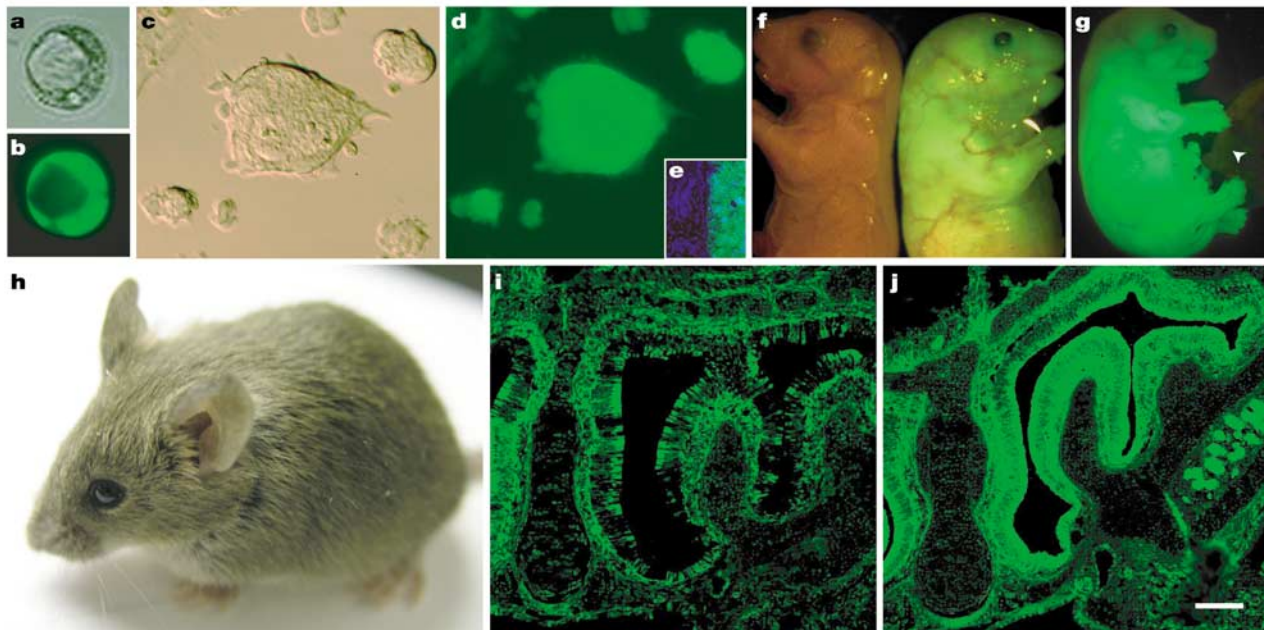
Finally, we performed two-colour *in situ* hybridization in chimaeric and clonal mice produced with green-fluorescent M71CZ ntES cells. Probes for *Cre* (to detect expression of the *M71-IRES-Cre* allele) or *M71* (to detect expression of the *M71-IRES-Cre* and wild-type *M71* alleles, but cross-hybridizing with the *M72* gene) reveal that only a very small subset of GFP-positive cells also express *Cre* (Fig. 5b) or *M71/M72* (Fig. 5c). Moreover, GFP-positive cells express other OR genes, such as *P2* (ref. 21) (Fig. 5d) and *MOR28* (ref. 23) (Fig. 5e). *P2* is expressed in a region of the epithelium that is different from *M71*, as can be assessed by two-colour *in situ* hybridization with *OCAM* (Fig. 5f). Green-fluorescent axons innervate multiple glomeruli, consistent with the expression of any of a variety of ORs in the OSNs (Fig. 5g). Thus, OSNs containing the same nucleus as once belonged to an

*M71*-expressing cell are not locked in to choose only *M71* for expression but can express other OR genes. More analyses will be required to screen for expression of other OR genes, and to quantify the probability of *M71* gene choice.

**Discussion**

The efficiency of ntES cell establishment is 1/63 successfully reconstructed oocytes or 1/7 cloned blastocysts in this study, which is comparable to 1/66 oocytes or 1/12 blastocysts in a survey of the mouse ntES literature with other differentiated cell types<sup>20</sup>.

In OSNs derived from OMP-Cre × Z/EG ntES cells, the expression of randomly selected genes of the OR repertoire would not be expected if the OSN used for nuclear transfer harboured a genomic alteration in OR gene *X* that instructed ntES-derived OSNs to choose and express invariably OR gene *X*. The argument is that pan-OSN expression of *X* would preclude expression of the rest of the OR repertoire by negative feedback<sup>28,29</sup>, and would organize the projection of axons to one or a few (gigantic) glomeruli in the olfactory bulb. However, there are several caveats: (1) The donor cells may have expressed OMP in the past but not at the time of observation, and may not correspond to mature OSNs. (2) Some OMP-positive cells may not express any OR gene. Single-cell reverse transcription (RT)-PCR of OSNs yields a cloned OR in 27–83% (average 36%) of cases<sup>5,30</sup>. The remaining OSNs are either false negative, or do not express any OR gene. (3) The hypothetical genomic alteration in OR gene *X* may be preserved upon nuclear transfer but not be sufficient for expression. As *X* is not known in advance, it can only be identified retrospectively if its expression is greatly increased. (4) Recent results have supported the notion of negative feedback of OR expression<sup>28,29</sup>, although it is difficult to distinguish it from negative selection against OSNs expressing more than one OR<sup>31</sup>. The mechanism of the presumptive negative feedback may depend on a normal process of OR gene choice. Negative



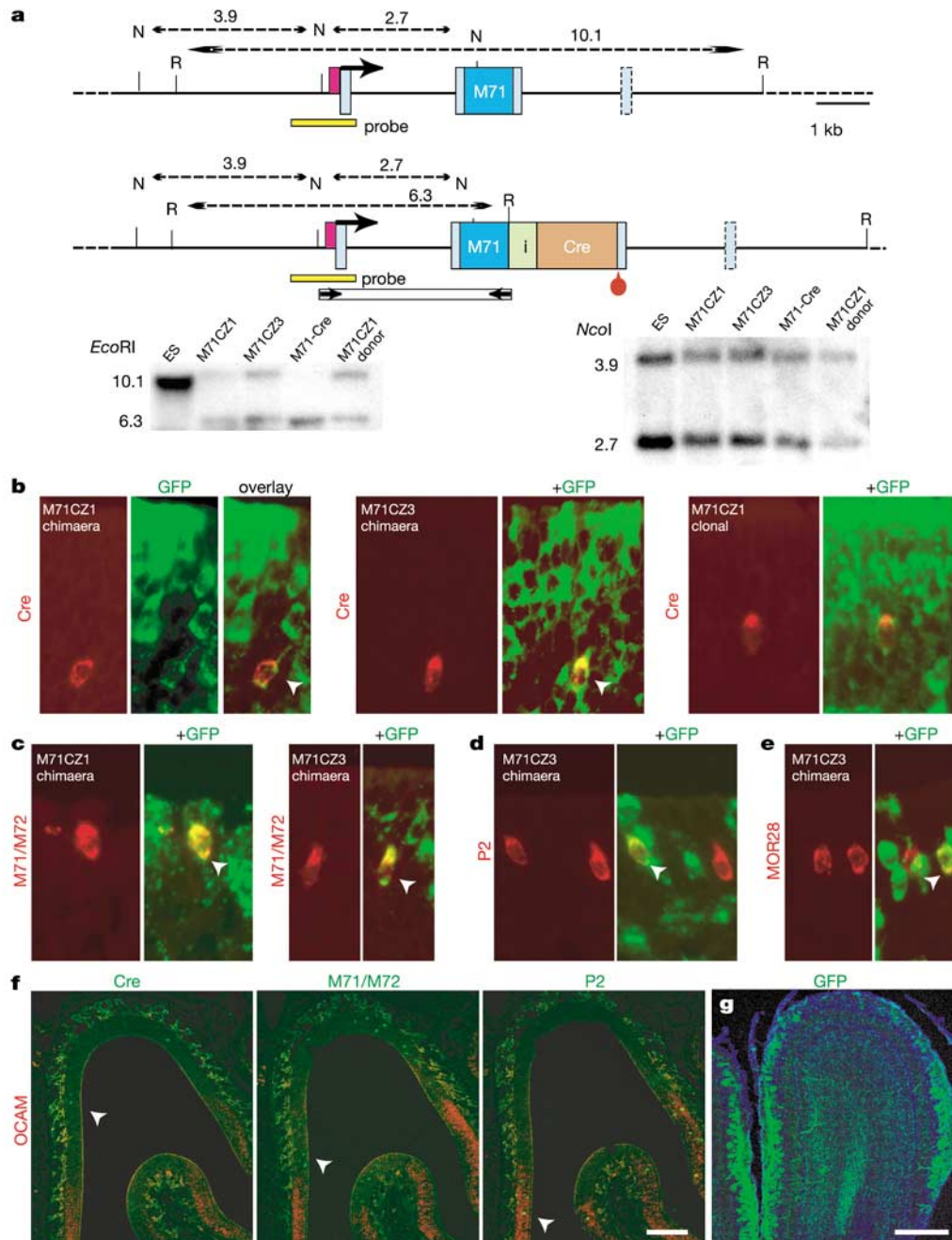
**Figure 4** ntES cell lines generated by nuclear transfer from permanently marked, *M71*-expressing OSNs. **a**, Blastocyst, bright-field image. **b**, Same blastocyst, fluorescent image. **c**, M71CZ1 ntES cell colonies in culture, bright-field image. **d**, Same cells, fluorescent image. All cells are strongly green fluorescent. **e**, Comparison of M71CZ2 (not green fluorescent, left) and M71CZ3 (green fluorescent, right). Counterstained with blue nuclear dye TOTO-3. **f**, Normal E19 mouse (left); chimaeric E19 mouse with M71CZ1 ntES cells (right). **g**, Clonal mouse produced by M71CZ1 ntES cell injection into tetraploid

blastocyst. Arrowhead indicates extra-embryonic tissues, which are host-derived and thus not green fluorescent. **h**, Clonal mouse Harvey at 3 weeks. This mouse was generated by injecting M71CZ3 ntES cells (agouti) into a B6D2F1 tetraploid blastocyst (black). **i**, Section through olfactory epithelium of chimaeric mouse produced by diploid blastocyst injection. Not all cells are green fluorescent. **j**, Section through olfactory epithelium of clonal mouse produced by tetraploid blastocyst injection. All cells are green fluorescent. Scale bar, 100 μm.

feedback may not occur when this choice is bypassed by pan-OSN expression of OR X. (5) OR X may be unable to prevent expression of the limited set of OR genes we tested.

To address these limitations, we carried out experiments with permanently marked OSNs that express a predefined OR, *M71*. Does the marking strategy accurately reflect the OR expressed at the

time of observation? The cross between *M71*-RFP, *M71*-IRES-Cre and *Z/EG* (Fig. 3b, c) demonstrates that once an individual OSN has chosen to express an *M71* allele, expression is irreversible. If switching to another OR gene, transient switching to the other *M71* allele, or biallelic *M71* expression were to occur, green-fluorescent cells would exceed the number of red-fluorescent cells, double-



**Figure 5** Genomic and phenotypic analysis of *M71CZ* ntES cell lines. **a**, Top, structure of wild-type *M71* locus. Middle, structure of *M71*-IRES-Cre targeted locus. Length of restriction fragments detected by the Southern blot probe (yellow) is indicated. White bar with arrows indicates sequenced region after amplification with long-range PCR. Red box is the minimal upstream region for expression in transgenic mice. R, *EcoRI*; N, *NcoI*. Bottom, Southern blot hybridization. For *EcoRI* digests, the wild-type and *M71*-IRES-Cre allele have bands of different sizes. Lane labelled 'M71CZ1 donor' is DNA from the tail of the mouse that was the nucleus donor for the M71CZ1 ntES cell line. **b-e**, Two-colour *in situ* hybridization of chimaeric or clonal mice generated by injection of *M71CZ* ntES cells. Probes in red are for *Cre* (detecting the *M71*-IRES-Cre allele) (**b**); *M71/M72* coding

region (**c**); *P2* (**d**); and *MOR28* (**e**). Probe in green in **b** is for *GFP*. Arrowheads in overlays indicate yellow colour, resulting from combined red and green fluorescence. *M71CZ1* chimaera, E19; *M71CZ3* chimaera, 2 weeks; *M71CZ3* clonal, E19. **f**, Two-colour *in situ* hybridization of a 2-week-old chimaera generated by injection of *M71CZ3* ntES cells. Probe in red is for *OCAM*, which is not expressed in the dorsal epithelium. As expected, *Cre* and *M71/M72* probes label cells (arrowheads) in the *OCAM*-negative, dorsal epithelium, whereas *P2* is co-expressed with *OCAM*. Scale bar, 200  $\mu$ m. **g**, Section of olfactory bulb of the same mouse as in **f**, counterstained with TOTO-3. Green-fluorescent axons project to multiple glomeruli. Scale bar, 500  $\mu$ m.

fluorescent cells would appear, and axons of some green-fluorescent cells (which actually express another OR gene) would project to glomeruli that are distinct from the M71 glomeruli. If a green-fluorescent cell were to cease expression of M71 and any other OR gene altogether, it would not be expected to survive very long.

These lineage-tracing experiments argue that an immature OSN does not alternate expression between the two M71 alleles before making its final choice. They further demonstrate that expression of M71 is irreversible during differentiation and throughout the lifetime of an OSN, at least up to the time of observation. From a practical point of view, they validate the use of the M71-IRES-Cre allele as a substitute for the M71-IRES-GFP or M71-IRES-tauGFP allele, with the crucial advantage of a permanent marker.

As outlined in ref. 18, the permanent marker strategy is essential in order to identify with certainty the origin of the donor nucleus that contributed to the generation of a particular cloned animal. Manipulation errors are unavoidable when success is rare and the target cell population is even rarer: in the third and critical set of mice (heterozygous M71-IRES-Cre, hemizygous Z/EG), only ~1/10,000 cells in dissociated cell preparations of epithelia is green fluorescent, and 1/52 nuclear transfers generated an ntES cell line. The permanent marker strategy provides a rigorous safeguard against accidental nuclear transfer with any of the remaining 99.99% of cells. Such mistakes would give the same, essentially negative, results (OR gene choice can be reset, and does not involve genomic alterations.) Using M71-IRES-GFP or M71-IRES-tauGFP mice would be inconclusive because such mistakes remain undetected.

Thus, we conclude that expression of the M71 OR gene is strictly monoallelic and irreversible: expression does not switch to the other M71 allele or to other OR genes during differentiation and throughout the lifetime of an OSN. M71 gene choice is not instructed by DNA rearrangements, assuming that these genomic alterations would have been preserved upon nuclear transfer and ntES cell derivation. Thus, OR gene expression is unlike that of immunoglobulin and T-cell receptor genes. □

Methods

Gene targeting

ES cells of cell line E14 (of 129 origin) were used<sup>32</sup>. OMP and M71 targeting vectors have been described<sup>21,25</sup>. The Cre coding sequence was excised from pBS185 (ref. 33). Pacl cassettes inserted were Cre for OMP and IRES-Cre along with a neo-selectable marker flanked by FRT sites. FNF. Flp-mediated excision of M71-Cre was carried out *in vivo* by crossing to Actin-*flp* transgenic mice and subsequent outcrossing of the *flp* transgene<sup>34</sup>. The ES clone name for OMP-Cre is F60 and for M71-Cre clone G12. M71-RFP (clone TD71-8) is the M71-IRES-tauRFP<sub>2</sub> mutation with the neo-selectable marker removed using ACNf by self-excision in the germ cells of male chimaeras<sup>35</sup>. RFP (dsRed-1)<sup>36</sup> is from Clontech; RFP<sub>2</sub> is a dsRed dimer linked by seven amino acids, VDPVAT. Mice are in a mixed 129 x C57BL/6 background. Z/EG mice<sup>22</sup> were purchased from the Jackson Laboratory.

Dissociation of OSNs

Olfactory epithelia were dissected from 2-week-old (OMP-Cre), and 2–4-week-old mice (M71-Cre), hemizygous for Z-E/G and heterozygous for the targeted mutation. Tissue was dissected in Ringer's solution, and dissociated with forceps and razor blades; no enzymes were used. Cells were washed three times with HEPES-CZB medium and suspended in HEPES-CZB medium containing 3% (w/v) polyvinylpyrrolidone<sup>36</sup>.

Nuclear transfer and embryo culture

This was performed as described<sup>12</sup>. Briefly, metaphase II-arrested oocytes were collected from superovulated B6D2F1 females (8–10 weeks) and cumulus cells were removed using hyaluronidase. Oocytes were cultured in CZB medium supplemented with 5.5 mM glucose at 37 °C under 5% CO<sub>2</sub> in air. Enucleation was performed in a droplet of HEPES-CZB medium containing 5 µg ml<sup>-1</sup> cytochalasin B using a blunt Piezo-driven pipette. After enucleation, the spindle-free oocytes were washed extensively and kept in CZB medium up to 2 h before nucleus injection. Green-fluorescent donor cells were picked up and aspirated in and out of the injection pipette to remove the cytoplasmic material. Each nucleus was injected into an enucleated oocyte using Piezo microinjection. The reconstructed oocytes were cultured in CZB medium for 1–3 h and then were activated for 5–6 h in calcium-free CZB medium containing 10 mM Sr<sup>2+</sup> and 5 µg ml<sup>-1</sup> cytochalasin B. Following activation, all reconstructed embryos were cultured in KSOM medium with amino acids at 37 °C under 5% CO<sub>2</sub> in air.

ES cell derivation

The reconstructed embryos were cultured in KSOM medium with amino acids for 3.5–4 days to reach the blastocyst stage. Expanded or hatched blastocysts were selected to establish ntES cell lines as described<sup>14</sup>. Each blastocyst was transferred into one well of a 96-well plate seeded with mouse-strain ICR embryonic fibroblast feeders in ES medium supplemented with 20% knockout serum replacement, 1,500 U ml<sup>-1</sup> leukaemia inhibitory factor (LIF) and 50 µM of the MEK1 inhibitor (PD98059) (ref. 37) after the zona pellucida was removed using acidic Tyrode's solution. After 4–7 days in culture, colonies were trypsinized and transferred to a 96-well plate with a fresh feeder layer in fresh medium. Clonal expansion of ntES cells proceeded from 48-well plates to 6-well plates with feeder cells and then to gelatinized 25-cm<sup>2</sup> flasks for routine culture in ES medium, with 15% fetal calf serum (FCS) and 1,000 U ml<sup>-1</sup> LIF.

Injection of ntES cells into blastocysts

Diploid blastocysts were collected from the uterus of E3.5 superovulated C57BL/6 females and kept in KSOM medium with amino acids until ntES cell injection. To obtain tetraploid blastocysts, two-cell embryos from E2 superovulated B6D2F1 females were electrofused to produce one-cell tetraploid embryos. Two-cell embryos were aligned by alternating current (a.c.) in 0.3 M mannitol solution, and a single direct current (d.c.) pulse of 1,000 V cm<sup>-1</sup> for 20 µs was applied. After electrofusion, embryos were returned to KSOM with amino acids and fused embryos were cultured for two days to reach the blastocyst stage. For blastocyst injection ES cells were trypsinized, resuspended in DMEM without LIF, and kept on ice. A flat tip microinjection pipette was used for ES cell injection. One hundred ES cells were picked up in the end of the injection pipette and injected into ten blastocysts. The blastocysts were kept in KSOM with amino acids until embryo transfer. Ten injected blastocysts were transferred into each uterine horn of 2.5-days-postcoitum pseudopregnant ICR females. Pregnant recipients with tetraploid embryos were subjected to caesarean section on day 19.5 of gestation.

Sequence analysis

We have shown that 161 base pairs (bp) upstream of the M71 transcription start site is sufficient for the recapitulation of M71 expression in transgenic mice (ref. 17, and our unpublished observations). Oligonucleotides were designed to amplify ±500 bp of the M71 transcription start site. Long-range PCR was performed from –241 bp until the IRES sequence, which immediately follows the M71 coding region, with oligonucleotides 5'-CGGCTC TGTTGATGCTAAATGTTTCATTGCC and 3'-GCGGAATTCCTAATTAACATCAAAGACTTTTC. PCR products were directly sequenced.

In situ hybridization

Antisense digoxigenin- and/or fluorescein-labelled complementary RNA probes prepared from cloned segments of complementary DNAs encoding GFP (full coding sequence), OMP (nucleotides (nt) 820–2891 from U01213), Cre (full coding sequence), M71 (nt 64–930 from AF281061), P2 (nt 54–948 from AF247657), MOR251-4 (nt 483–895 from NG002083), MOR28 (ref. 23), OCAM (nt 496–2002 from AF001287). Sections (10 µm) were cut from the olfactory epithelium. Two-colour fluorescent *in situ* hybridization was performed as described<sup>38,39</sup>. The digoxigenin-labelled probe was visualized with HNPP/FAST RED, and the fluorescein-labelled probe was visualized with Alexa 488. Sections were analysed using a confocal microscope Zeiss LSM 510.

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