

# Mice cloned from olfactory sensory neurons

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**Cloning by nuclear transplantation has been successfully carried out in various mammals, including mice. Until now mice have not been cloned from post-mitotic cells such as neurons. Here, we have generated fertile mouse clones derived by transferring the nuclei of post-mitotic, olfactory sensory neurons into oocytes. These results indicate that the genome of a post-mitotic, terminally differentiated neuron can re-enter the cell cycle and be reprogrammed to a state of totipotency after nuclear transfer. Moreover, the pattern of odorant receptor gene expression and the organization of odorant receptor genes in cloned mice was indistinguishable from wild-type animals, indicating that irreversible changes to the DNA of olfactory neurons do not accompany receptor gene choice.**

The chromosome is a dynamic structure that undergoes complex changes that underlie the development and differentiated function of an organism. Alterations in chromosome structure include variation in the complement of regulatory proteins, covalent modifications in chromatin proteins or DNA, and in rare instances, DNA rearrangements<sup>1</sup>. The extent to which such chromosomal changes are reversible can be discerned by cloning experiments involving nuclear transfer. Thus far, cloning experiments using nuclei from post-mitotic cells that have irreversibly exited the cell cycle as part of their programme of differentiation have not generated viable embryos or mice<sup>2,3</sup>. These observations have led to the suggestion that post-mitotic cells might be refractory to epigenetic reprogramming or alternatively might have acquired changes in their DNA that could limit their developmental potential<sup>4,5</sup>.

Directed DNA rearrangements are rarely observed as part of a normal differentiation programme and, in vertebrates, they have been described only for the generation of the diverse repertoire of antibodies and T-cell receptors<sup>6–8</sup>. Several observations have suggested that post-mitotic neurons may also use irreversible DNA alterations to generate diversity. First, a population of neurons undergoes apoptosis in mice bearing mutations in the double-strand-break DNA-repair enzymes, which are also required for DNA rearrangements in lymphocytes<sup>9,10</sup>. Second, cortical neurons exhibit a higher incidence of aneuploidy than other cell types, although the functional significance of these changes is unknown<sup>11,12</sup>. These observations and the inability to clone mice from neuronal nuclei have led to models in which the DNA of post-mitotic neurons might undergo rearrangements to supply additional genetic diversity that may enhance neural function<sup>4,5,13–15</sup>.

One particularly clear example of neuronal diversity is provided by the olfactory sensory epithelium. In the mouse, each of the 2,000,000 cells in the olfactory epithelium expresses only one of about 1,500 odorant receptor genes, such that the functional identity of a neuron is defined by the nature of the receptor it expresses<sup>16</sup>. Thus, the sensory epithelium consists of at least 1,500 neuronal types. The pattern of receptor expression is apparently random within one of four zones in the epithelium, suggesting that

the choice of receptor gene may be stochastic<sup>17,18</sup>. One mechanism to permit the stochastic choice of a single receptor could involve DNA rearrangements<sup>19,20</sup>.

Here we report the generation of fertile mouse clones by transferring the nuclei of post-mitotic olfactory neurons into enucleated oocytes. Thus, a post-mitotic nucleus can re-enter the cell cycle and be reprogrammed to totipotency. The DNA of mice derived from sensory neurons reveals no evidence for rearrangements of the expressed olfactory receptor gene. The pattern of receptor expression in these mice was indistinguishable from that of wild-type animals, indicating that irreversible changes in DNA do not accompany olfactory receptor gene choice.

## Genetic marking of olfactory sensory neurons

Less than 1% of nuclear transfers result in the production of an embryonic stem (ES) cell line or live animal<sup>1</sup>. It is therefore difficult to identify with certainty the origin of the donor nucleus that contributed to the generation of a particular cloned animal<sup>2</sup>. This problem is particularly apparent in the olfactory epithelium, which contains mature sensory neurons intermingled with stem cells, neural progenitors and support cells. Therefore, we generated mice in which the endogenous olfactory marker protein (OMP) promoter drives simultaneous expression of OMP and Cre recombinase by inserting an internal ribosome entry site (IRES)-Cre cassette 3' of the OMP stop codon. OMP is expressed only in mature olfactory sensory neurons (OSNs). When OMP-IRES-Cre mice are crossed to a reporter mouse strain (Z/EG), Cre expression catalyses the excision of a transcriptional stop sequence and results in green fluorescent protein (GFP) expression solely in mature OSNs (Fig. 1a and Methods). Transfer of marked OSN nuclei into oocytes should generate embryos in which every cell expresses GFP, as the Z/EG reporter uses the ubiquitous actin promoter.

In mice bearing the OMP-IRES-Cre allele and the Z/EG reporter gene, GFP expression was observed only in the most mature OSNs. Sections through the entire olfactory epithelium of several adult mice revealed GFP expression in the regions that contain mature OSNs (Fig. 1b–f). Cell counts revealed no GFP<sup>+</sup> cells in either the basal cell layers, where immature progenitors and stem cells reside, or in the apical support cell layer (0 of 835 GFP<sup>+</sup> cells)<sup>21,22</sup>. Double

immunostaining for GFP and Cre protein revealed that all GFP<sup>+</sup> cells with visible nuclei also expressed Cre recombinase (599 of 599) (Fig. 1c). In addition, none of the MASH-1<sup>+</sup> basal cell precursors of OSNs expressed GFP (0 of 350 MASH-1<sup>+</sup> cells) (Fig. 1d). These results showed that GFP expression was restricted to mature sensory neurons.

We confirmed that the GFP<sup>+</sup> mature OSNs were post-mitotic by injecting mice with 5-bromodeoxyuridine (BrdU) and staining the olfactory epithelium with anti-BrdU and GFP antibodies (Fig. 1e). BrdU incorporation was observed in the more basal GFP-negative layers as well as in rare support cells in the apical layers. Examination of more than 3,000 GFP<sup>+</sup> cells never revealed a GFP<sup>+</sup> BrdU<sup>+</sup> cell. In a separate experiment, labelling with an antibody specific to Ki67, a protein restricted to the nuclei of dividing cells, revealed no staining coincident with GFP<sup>23,24</sup> (Fig. 1f). These data show that GFP<sup>+</sup> cells in the olfactory epithelium of donor animals are mature post-mitotic OSNs.

### Cloning mice from neuronal nuclei

We asked whether the nucleus of a post-mitotic OSN could re-enter the cell cycle and direct preimplantation development. We dissociated the olfactory epithelium and picked GFP-expressing OSNs for nuclear transfer into enucleated oocytes (Fig. 2a)<sup>25–29</sup>. Of the 352 embryos generated, 48 (14%) developed to the blastocyst stage (Table 1). All blastocysts expressed GFP, demonstrating that they were derived from the mature OSN donor nuclei (Fig. 2b). We confirmed that Cre expression in the OSNs caused constitutive GFP expression after nuclear transfer by generating blastocysts with GFP-negative cells from the donor mice. As expected, none of these blastocysts expressed GFP, demonstrating that Cre is not aberrantly expressed as a result of nuclear transfer or subsequent cloning procedures.

The 48 GFP<sup>+</sup> blastocysts were used to generate ES cell lines and three gave rise to colonies that resembled ES cells (OSN1–3). All three lines expressed GFP, and Southern blotting confirmed the predicted genomic rearrangement at the Z/EG locus (Fig. 2c and data not shown). When the OSN2 and OSN3 ES cells were injected into diploid blastocysts, they contributed extensively to all tissues of

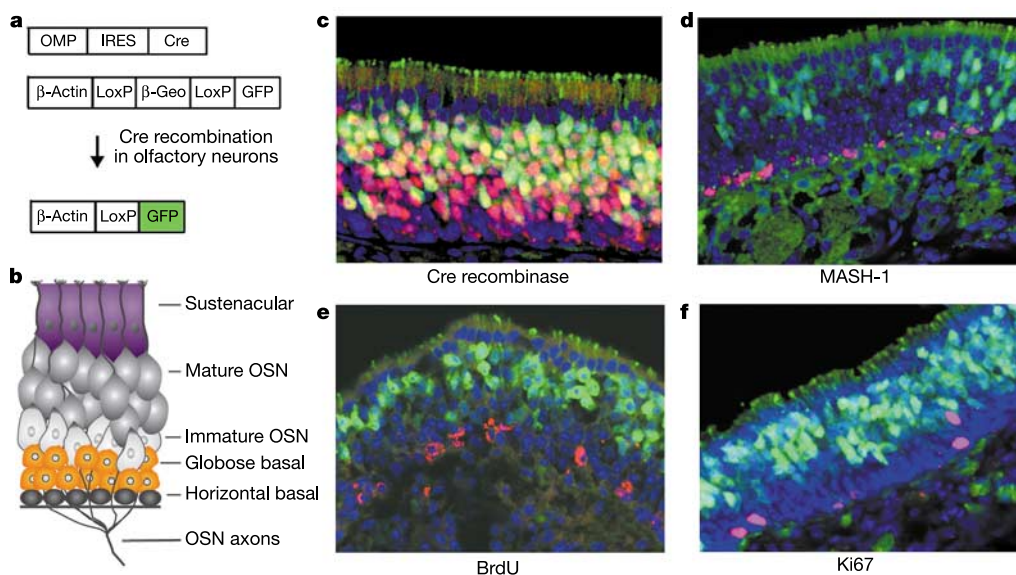
the resulting chimaeras, including the germ line (Fig. 2i, j and data not shown).

We injected these cloned ES cells into tetraploid blastocysts to perform the most stringent test of their developmental potency (Table 2). Tetraploid embryo complementation generates an ‘ES-fetus’ in which all embryonic lineages are derived from the injected ES cells, whereas the extraembryonic lineages develop from the host blastocyst<sup>29,30</sup>. Both OSN2 and OSN3 ES cells gave rise to embryos of embryonic day (E)19.5 that expressed GFP ubiquitously (Table 2 and Fig. 2d). Mice derived from OSN3 survived to adulthood, were fertile and were overtly normal. Histological examination of serial sections through their brains revealed no obvious abnormalities (data not shown). These results indicate that the genome of a post-mitotic, terminally differentiated neuron can be reprogrammed after nuclear transfer and direct the development of all embryonic lineages.

### Odorant receptor expression in cloned mice

We examined the patterns of receptor gene expression in the olfactory epithelium of the cloned mice generated by tetraploid complementation. If irreversible genetic rearrangements are required for receptor choice we might expect an altered profile of receptor expression in animals cloned from an OSN. In the simplest model, a rearrangement involving one receptor gene might persist in all neurons such that all neurons will express the same receptor. This scenario was observed in mice derived from a B-cell nucleus, in which all B cells expressed the same immunoglobulin gene<sup>28</sup>. We therefore asked whether sensory neurons from cloned mice express a single receptor or a repertoire of receptor genes. We performed *in situ* hybridization on the olfactory epithelium of mice derived from OSN3 ES cells using probes specific for seven different odorant receptors (Fig. 2e–h and data not shown). The pattern of expression of all seven receptors was indistinguishable from control mice, excluding a simple model of gene rearrangement that would result in the expression of a single receptor gene in all OSNs.

As a neuron expresses a receptor from only one of the two alleles, it remained possible that a rearranged receptor gene expressed in the donor nucleus would be expressed in only half of the sensory



**Figure 1** Genetically marking post-mitotic OSNs. **a**, Donor animals carry one OMP-IRES-Cre allele and one copy of the Z/EG Cre reporter transgene. Cre expression in OSNs catalyses excision of the  $\beta$ -geo/stop cassette, resulting in selective GFP expression and genetic marking of mature OSNs. Thus, GFP<sup>+</sup> neurons could be selectively chosen for nuclear transfer. **b**, Schematic diagram of the approximate laminar distribution of cell

types in the mouse olfactory epithelium. **c–f**, Three-colour immunofluorescence staining. Blue, nuclear marker TOTO-3; green, GFP protein fluorescence (**c, d**) or antibody to GFP protein (**e, f**); red, antibodies to Cre recombinase (**c**), progenitor-specific marker MASH-1 (**d**), markers of dividing cells, BrdU (**e**) and Ki67 (**f**).

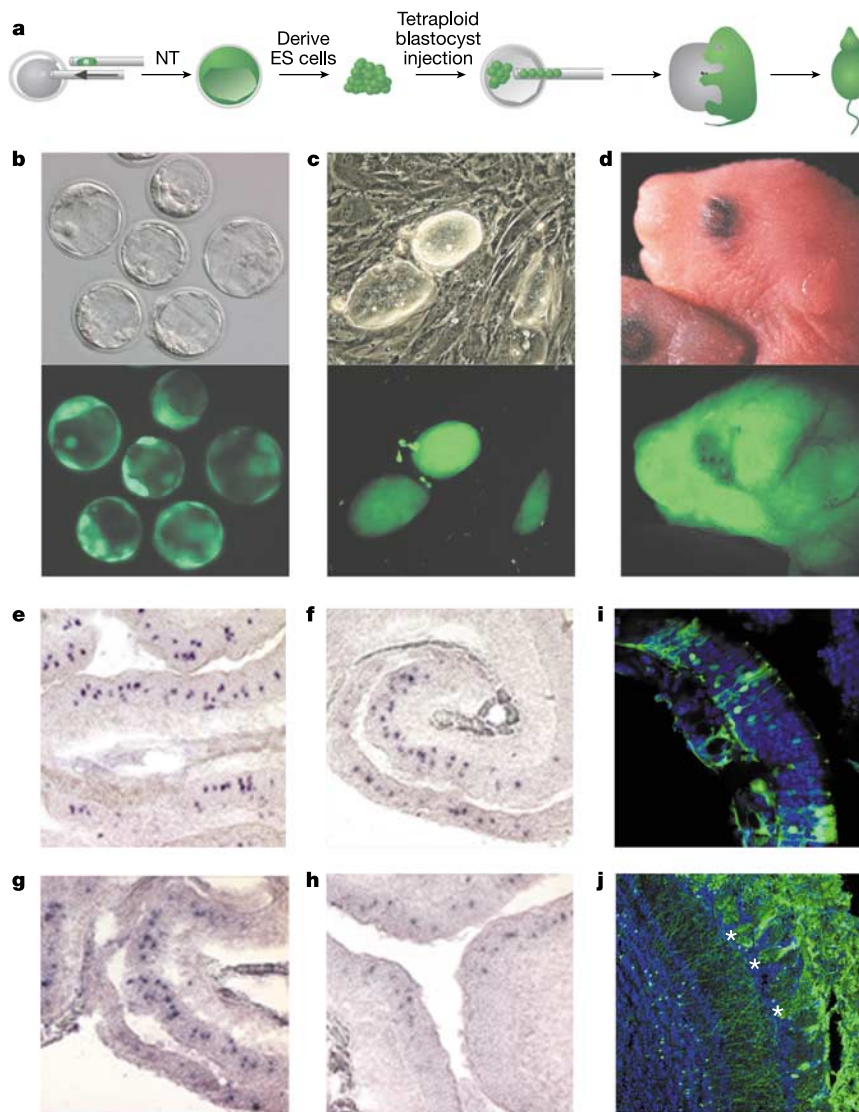
neurons. We therefore analysed the repertoire of receptors expressed by polymerase chain reaction with reverse transcription (RT-PCR) to determine whether a single receptor transcript was enriched in the epithelium of OSN-derived mice. RNA from the olfactory epithelium of cloned animals was used in RT-PCR reactions with degenerate primers that recognize conserved motifs present in the majority of odorant receptors. Forty-four PCR products were cloned and restriction digest analysis indicated that they encoded 38 different receptors. Sequence analysis of 20 of these PCR clones revealed that they encoded 20 different receptors, which were located in seven clusters on six different chromosomes. This suggests that a single odorant receptor did not predominate in the olfactory epithelium of mice derived from OSN nuclei.

One additional assay for the diversity of odorant receptor expression is based on the observation that neurons expressing a given receptor, although randomly distributed within a zone of the epithelium, converge on two spatially invariant loci or glomeruli in the olfactory bulb<sup>18,31</sup>. If half of the OSNs from cloned mice

expressed the same receptor, then their axonal projections should preferentially innervate a small set of glomeruli. Analysis of the olfactory bulb of chimaeric mice produced with OSN2 ES cells revealed that the OSN2-derived GFP<sup>+</sup> cells innervated most, if not all glomeruli, with no glomerulus receiving predominant input (Fig. 2i, j). These results show that the sensory neurons of mice cloned from an OSN that had expressed a single receptor can express a large repertoire of odorant receptor genes.

**Mice cloned from neurons expressing the P2 receptor**

The previous experiments suggest that irreversible rearrangements are not required for receptor gene expression. Because these experiments did not allow the prospective identification of the receptor gene expressed by the donor OSN nucleus, we were unable to examine its pattern of expression or its DNA sequence in the cloned mice. Neurons that express the P2 odorant receptor were marked by introducing an IRES directing the translation of GFP into the 3' untranslated region of the P2 gene (P2-IRES-GFP)<sup>32</sup>.



**Figure 2** Mice derived from OSN nuclei. **a**, Strategy to generate cloned ES cell lines and OSN-derived mice by tetraploid complementation with cloned ES cells. **b, c**, Bright-field and fluorescence images of nuclear transfer blastocysts (**b**) and OSN3 ES cells (**c**) produced from OSN nuclei. **d**, A P0.5 mouse produced by tetraploid complementation with OSN3 ES cells (top) and a P0.5 C57/B6 control (bottom). **e-h**, *In situ* hybridization on

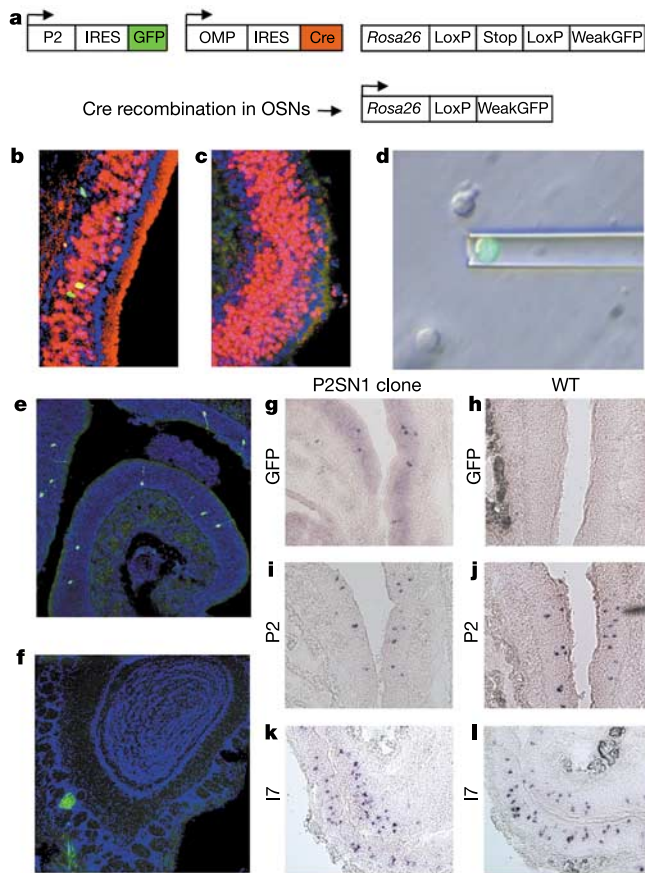
the olfactory epithelium of mice wholly derived from OSN3 ES cells with probes for odorant receptors P2 (**e**), M50 (**f**), I7 (**g**) and M71 (**h**). **i, j**, Contribution of GFP<sup>+</sup> OSN2 ES cells to the olfactory epithelium (**i**) and olfactory bulb (**j**) of chimaeras generated by injecting OSN2 ES cells into diploid blastocysts. Several representative glomeruli are demarcated with asterisks.

Table 1 Production of ES cell lines and mice by nuclear transfer

Donor cells	Oocytes surviving (% injected)	Oocytes activated (% surviving)	Two-cell embryos to oviduct (% oocytes activated)	Blastocysts (% oocytes activated)	ES cell lines (% oocytes activated)	Alive at term (% into oviduct)
OSNs	508 (88)	352 (69)	—	48 (14)	3 (1)	—
P2 OSNs	261 (95)	141 (54)	—	18 (13)	2 (1)	—
P2SN1 ES	50 (95)	38 (76)	14 (37)	—	—	1 (7)
P2SN2 ES	47 (90)	37 (79)	18 (49)	—	—	1 (6)

Table 2 Mice produced from sensory neurons by tetraploid embryo complementation

ES cell line	Receptor expressed	Tetraploid blastocysts injected	Mice alive at term (% injected)	Breathing normally (% injected)	Cross-fostered	Survival to maturity (% fostered)
OSN1	?	137	0	0	0	0
OSN2	?	160	1 (1)	0	0	0
OSN3	?	273	49 (18)	29 (11)	21	17 (81)
P2SN1	P2	89	16 (18)	16 (100)	13	12 (92)
P2SN2	P2	151	31 (21)	28 (90)	28	22 (78)



**Figure 3** Mice produced from OSNs expressing the P2 odorant receptor. **a**, Strategy to label P2 sensory neurons with GFP and genetically mark mature OSNs. **b, c**, Immunofluorescence staining with nuclear marker TOTO-3 (blue), GFP fluorescence (green) and antibodies to Cre recombinase (red) of the olfactory epithelium of donor animals heterozygous for the P2-IRES-GFP, OMP-IRES-Cre and *Rosa26*-LoxP-Stop-LoxP-weak GFP alleles (**b**) or of control animals heterozygous for the OMP-IRES-Cre and *Rosa26*-LoxP-Stop-LoxP-weak GFP alleles (**c**). **d**, Bright-field and fluorescence merged image of picking a P2-IRES-GFP<sup>+</sup> neuron from the dissociated olfactory epithelium of a donor for nuclear transfer. **e, f**, GFP fluorescence (green) and TOTO-3<sup>+</sup> nuclei (blue) visualized in sections of the olfactory epithelium (**e**) and olfactory bulb (**f**) of an animal generated by tetraploid complementation with P2SN1 ES cells. **g–l**, *In situ* hybridization on olfactory epithelium sections derived entirely from P2SN2 ES cells (**g, i, k**) and a wild-type control (**h, j, l**) with probes for GFP (**g, h**), P2 receptor (**i, j**) and I7 receptor (**k, l**).

These mice were crossed with strains carrying both the OMP-IRES-Cre alteration and a reporter allele in which the *Rosa26* promoter is separated from a weak GFP gene by a LoxP-flanked transcriptional terminator<sup>33</sup> (Fig. 3a). Mice carrying the P2-IRES-GFP, OMP-IRES-Cre and *Rosa26*-LoxP-Stop-LoxP-weak GFP reporter genes exhibited intense GFP fluorescence in the approximately 0.1% of the OSNs that expressed the P2-IRES-GFP allele, whereas all other neurons appeared dark (Fig. 3b). *Rosa26*-driven GFP was present in mature OSNs, but the GFP signal was too weak to be detected by direct fluorescence. No green cells were observed in animals that contained only the OMP-IRES-Cre and *Rosa26*-LoxP-Stop-LoxP-weak GFP genes (Fig. 3c). Evidence for Cre-mediated excision of the transcription terminator and the resulting expression of weak GFP in mature OSNs was detected by amplifying the signal with GFP antibodies (data not shown).

Fluorescent cells expressing the P2-IRES-GFP allele were picked and their nuclei were injected into enucleated oocytes (Fig. 3d). Eighteen blastocysts developed from 141 reconstructed oocytes generating two ES cell lines: P2SN1 and P2SN2 (Table 1). PCR analysis revealed Cre-mediated excision at the *Rosa26* locus in both cell lines, indicating their origin from cloned nuclei of post-mitotic OSNs (Supplementary Fig. 1). Tetraploid embryos injected with P2SN1 and P2SN2 cells resulted in the birth of multiple viable pups that survived to adulthood (Table 2). These mice exhibited no gross anatomic or behavioural abnormalities and were fertile. PCR analyses of non-neuronal tissues revealed recombination at the *Rosa26* reporter allele, indicating that the cloned mice were derived from mature OMP<sup>+</sup> OSNs (data not shown). In addition, we detected the weak *Rosa26*-driven GFP expression by *in situ* hybridization in both the olfactory epithelium and non-neuronal tissues, demonstrating genetic activation of the reporter (Fig. 3g, h and data not shown).

The expression pattern of P2-IRES-GFP in clones was indistinguishable from that of control donor animals. Approximately 0.1% of the neurons expressed GFP at a high level within zone II of the epithelium (Fig. 3e). The GFP-expressing neurons projected axons to one medial and one lateral glomerulus in the olfactory bulb (Fig. 3f). Moreover, the P2-IRES-GFP allele was expressed at a frequency approximately equal to that of the unmodified P2 allele. *In situ* hybridization was performed on sections through the olfactory epithelium with RNA probes specific to GFP- and P2-coding sequences (Fig. 3g–j). The number of GFP-expressing cells in one section was roughly 50% of the number of P2-expressing cells found in neighbouring sections ( $45 \pm 14\%$ , s.d.), revealing no preference for the expression of the P2-IRES-GFP allele. Furthermore, *in situ* hybridization with probes specific to six additional

olfactory receptors revealed similar expression patterns in control and cloned mice (Fig. 3k, l and data not shown).

We also performed Southern blotting, PCR and genome sequencing to examine the organization of the P2-IRES-GFP allele in the chromosome of cloned mice in an effort to detect potential DNA rearrangement events. If choice of a single receptor gene involved gene conversion into a single active locus, cells expressing the P2-IRES-GFP allele might contain a second copy of this allele at the active locus. Southern blotting to distinguish the modified P2-IRES-GFP allele from the unmodified endogenous P2 allele in DNA from control and cloned mice revealed only two bands of equal intensity, suggesting that gene conversion into an active locus does not accompany olfactory receptor gene choice (Supplementary Fig. S2). In addition, Southern blot analyses using multiple DNA probes to examine the organization of about 60 kilobases (kb) of DNA 5' and 3' of the P2 coding sequence failed to detect any differences between donor, control and cloned animals (Supplementary Fig. S3). Sequencing of 10 kb 3' of the P2 coding sequence showed that the P2-IRES-GFP allele was identical in donor, control and cloned mice (data not shown). These results suggest that irreversible changes in DNA do not accompany choice of the P2 odorant receptor gene.

### Totipotency of neuronal nuclei

The two-step cloning procedure used to produce mice from neuronal nuclei generates mice in which the neuronally derived ES cells give rise to all embryonic tissues, whereas cells from the tetraploid host blastocyst contribute to the embryonic trophoblast<sup>30</sup>. Thus, neither this work nor the cloning of lymphocytes via an ES cell intermediate<sup>28</sup> demonstrated the totipotency of a nucleus from a terminally differentiated cell<sup>34</sup>. To demonstrate totipotency of mature OSN nuclei, we transplanted nuclei from P2SN1 and P2SN2 ES cells into enucleated oocytes<sup>29</sup>. The resulting embryos were cultured for 24 h and transferred to pseudopregnant recipients (Table 1). Upon caesarean section of the recipients, we recovered full-term pups from both the P2SN1 and P2SN2 cell lines. These pups had enlarged placentas (P2SN1, 0.35 g; P2SN2, 0.40 g) but displayed no overt anatomical or behavioural abnormalities, were fertile and survived to adulthood, consistent with previous cloning experiments<sup>29</sup>. These observations demonstrate that nuclei of terminally differentiated olfactory neurons can be reprogrammed to totipotency, directing development of both embryonic and extra-embryonic lineages.

### Discussion

We have asked whether the nucleus of a post-mitotic olfactory sensory neuron can re-enter the cell cycle and undergo reprogramming to direct development of a mouse. ES cell lines were generated from OSN nuclei at frequencies similar to those obtained with differentiated lymphoid cells that can be induced to proliferate under physiological conditions<sup>2</sup>. Thus the mechanisms that lead to the cell-cycle exit and irreversible mitotic arrest that accompany neural differentiation do not result from irreversible epigenetic or genetic events that would interfere with nuclear totipotency.

The differentiation of neurons requires that neural progenitors exit the cell cycle before a restriction point late in G1. This decision is governed by a complex balance of cell-cycle regulators and proneural genes that drive cells into G0 and prevent them from progressing beyond the restriction point<sup>35</sup>. Although most cells that enter G0 can re-enter the cycle on appropriate stimulation, neurons normally undergo an irreversible mitotic arrest. Whatever mechanisms keep neurons in a post-mitotic state, our experiments demonstrate that they can be overcome in the environment of the egg.

The nervous system contains a diverse array of neural cell types and this diversity is reflected by distinct patterns of gene expression in different neurons. The regulation of gene expression by DNA

rearrangements is rare but this mechanism has nonetheless been suggested to explain the diversity inherent in complex nervous systems<sup>36</sup>. DNA recombination events provide *Saccharomyces cerevisiae*, trypanosomes and lymphocytes with a mechanism to stochastically express one member of a set of genes that modulate cellular interactions with the environment. One attractive feature shared by gene rearrangements in trypanosomes and lymphocytes is that gene choice by recombination is a random event. Cells that undergo correct or successful rearrangements are then afforded a selective survival advantage. The stochastic rearrangement of one gene from a gene family and subsequent selection could also provide a mechanism to generate the vast diversity of neuronal cell types. In this manner, neurons with subtly different genotypes would exhibit the array of neuronal phenotypes required for a functioning nervous system.

The olfactory sensory epithelium provides a clear example of neuronal diversity, and it has been suggested that this diversity is generated by stochastic DNA rearrangement events<sup>19,20</sup>. However, efforts to examine the DNA of OSNs expressing a given receptor have been seriously hampered by the inability to obtain homogeneous populations of neurons or clonal cell lines in which each cell expresses the same receptor. We have addressed this problem by generating cloned ES cell lines and mice derived from the nuclei of olfactory sensory neurons expressing the P2 receptor. Analyses of the sequence and organization of the DNA surrounding the expressed P2 allele from cloned ES cells and mice revealed no evidence for either gene conversion or local transpositions at the P2 locus. These results concur with fluorescence *in situ* hybridization studies showing that gene conversion into an active locus is an unlikely mechanism for odorant receptor expression<sup>37</sup>. In addition, the pattern of receptor gene expression in the sensory epithelium of cloned mice was wild type; multiple odorant receptor genes are expressed without preference for the P2 allele expressed in the donor nucleus.

These data demonstrate that the mechanism responsible for the choice of a single odorant receptor gene does not involve irreversible changes in DNA. More dynamic, reversible recombination events might accompany odorant receptor gene choice, but this is unlikely given the current data. Our results, in combination with experiments showing that the expression of various odorant receptor transgenes is influenced by proximal sequence elements, are consistent with epigenetic models of odorant receptor choice<sup>37-42</sup>. In a broader context, the generation of fertile cloned mice that are anatomically and behaviourally indistinguishable from wild-type mice indicates that olfactory sensory neurons do not undergo other irreversible DNA rearrangements that would interfere with either the development or function of the nervous system during adult life. □

### Methods

#### Preparation of donor neurons

Olfactory epithelia were dissected in L15 medium (Gibco) at 4 °C, then chopped into small pieces and incubated with type IV collagenase at 1 µg ml<sup>-1</sup> at 37 °C for 15 min with occasional vigorous shaking. The collagenase digestion was stopped by addition of DMEM plus 10% FBS. Cells were spun down and resuspended in trituration medium (PBS plus 30% glucose plus 10% FBS plus penicillin/streptomycin) and triturated with several widths of pipette tips to produce single cell suspensions. Cells were pelleted and resuspended in L15/10% FBS before picking.

#### ES cell lines and tetraploid embryo complementation

Production of ES cell lines from nuclear transfer embryos was exactly as described<sup>28</sup>, and generation of mice by tetraploid embryo complementation was exactly as described<sup>29</sup>.

#### Generation of cloned embryos and mice

Production of cloned embryos by nuclear transfer was essentially as described<sup>25,29</sup> except that only GFP<sup>+</sup> cells from the two donor populations, as identified by epifluorescence, were picked and used for nuclear transfer.

#### *In situ* hybridization and immunohistochemistry

Animals were killed by approved methods and tissues of interest were either fresh frozen in

OCT or fixed for 2–12 h in 4% PFA/1 × PBS, then washed and equilibrated in 30% sucrose before freezing. Frozen sections (from 20–30 μM) were placed on slides and standard immunohistochemistry and digoxigenin-labelled probe *in situ* hybridization protocols were used<sup>32</sup>. We used the following antibodies: rabbit anti-GFP antibody (Molecular Probes), rabbit anti-Cre recombinase antibody (Novagen), goat anti-Ki67 antibody (Santa Cruz Biotechnology), rat anti-BrdU antibody (abcam) and rabbit anti-MASH-1 antibody (a gift of J. Johnson).

### BrdU labelling and visualization

Mice were injected intraperitoneally with a 5 mg ml<sup>-1</sup> solution of BrdU in PBS every 2 h for 12 h. Each mouse received BrdU at 100 μg g<sup>-1</sup> body weight for each injection. Two hours after the last injection mice were killed and tissues were fixed for 2 h in 4% PFA and PBS, washed and sucrose protected, and frozen. To permit simultaneous visualization of BrdU and GFP, sections were first stained with anti-GFP antibody then fixed in 4% PFA/PBS for 15 min, then washed, treated with 4 M HCl/0.1% Triton X-100 for 10 min, washed and stained with rat anti-BrdU followed by the appropriate secondary antibodies.

### Gene targeting and generation of donor mouse lines

The OMP-IRES-Cre mouse line was generated as described previously except that the Cre recombinase gene sequence was inserted in place of the tTA sequence<sup>32</sup>. An IRES directing the translation of Cre recombinase was introduced into the 3' untranslated region of the OMP locus by homologous recombination in ES cells. Transgenic mice were generated and crossed with a strain bearing the Z/EG reporter transgene. The Rosa26-LoxP-Stop-LoxP-GFP line was generated as described previously except that the GFP sequence replaced the CFP and YFP sequences in the Rosa26 cassette<sup>33</sup>.

### Southern blotting, PCR and RT-PCR

Southern blots and PCR screening of ES cells and tails used standard methods<sup>33</sup>. RT-PCR was performed on RNA isolated from mouse olfactory turbinates that had been frozen in Trizol (Invitrogen), and then treated as per the manufacturer's protocol. RNA was reverse transcribed using the Superscript II kit (Invitrogen) and complementary DNA was subjected to PCR using standard conditions.

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