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Sequestration within nuclear chromocenters is not a requirement for silencing olfactory receptor transcription in a placode-derived cell line

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Mouse olfaction depends on specialized olfactory sensory neurons (OSNs) that each express only one olfactory receptor protein from among a family of >1000 olfactory receptor (OR) genes encoded in the genome. To investigate epigenetic mechanisms underlying monogenic OR expression, we characterized the nuclear organization of OR loci in an olfactory placode-derived cell line (OP6) derived from a pre-neuronal cell along the OSN lineage. OR loci are significantly enriched within nuclear chromocenters in these cells as compared with control loci tested. However, we observe variability in chromocenter occupancy among different OR loci and from cell-to-cell, suggesting that these associations are transient or context dependent. The lamin B receptor (LBR), whose downregulation is necessary for aggregation of chromocenters and OR genes in mature OSNs, exhibits an unusual non-peripheral expression pattern in OP6 nuclei; upon further OP6 cell differentiation, LBR expression is lost and chromocenters begin to aggregate. However, neither undifferentiated nor differentiated OP6 cells sequester OR genes within the chromocenters, despite the establishment of monogenic OR expression in these cells. These results indicate that sequestration of competing OR loci is not a requirement for monogenic OR expression in OP6 cells, and could indicate that the initial establishment of monogenic OR expression during OSN differentiation in vivo occurs prior to recruitment of OR genes into chromocenters.

Introduction

The proper function of the mammalian olfactory system depends on the specialization of individual sensory neurons, each of which becomes specialized for a narrow range of odorant chemistry by expressing only one type of olfactory receptor (OR) protein.[1-4] The mouse genome encodes >1000 OR genes, which are clustered at >50 genome locations and on multiple chromosomes (reviewed in ref. 5). Thus, each sensory neuron in the developing olfactory epithelium is tasked with transcribing one OR gene, while silencing neighboring OR genes within the same cluster, as well as silencing a large set of OR loci in trans, including the other parental allele.

The regulation of histone marks and nuclear positioning is a common theme in the establishment of mutually exclusive transcription for several gene families.[6-11] The nucleus is organized such that heterochromatin and euchromatin form distinct segregated domains. Facultative heterochromatin contains compacted DNA enriched for reversible repressive marks, such as H3K27 methylation, and is commonly sequestered to the nuclear periphery (reviewed in refs. 12 and 13). Constitutive heterochromatin contains major satellite and other repetitive DNA that is highly compacted and enriched in stable repressive marks, such as H3K9me3 and H4K20me3, and is commonly sequestered within nuclear chromocenters (reviewed in refs. 14 and 15). Importantly, RNA polymerase and heterochromatic compartments are mutually exclusive (ref. 16, herein), so genes that occupy the nuclear periphery or chromocenters will generally be restricted from accessing transcriptional factories.

A model for the regulation of mutually exclusive OR transcription has recently gained momentum in which all OR gene loci are initially silenced followed by the stochastic de-repression of a single OR locus. Support for this model includes: (1) repressive histone-3, lysine-9 trimethylation (H3K9me3) marks are established early at OR loci in the olfactory sensory neuronal (OSN) lineage and prior to OR gene selection, and these repressive marks are later removed only on the transcribed OR allele[17]; (2) deletion of the lysine-specific demethylase-1 (LSD1) thought to be involved with the singular removal of H3K9 methylation marks at the transcribed OR allele globally impairs OR expression, suggesting that H3K9 demethylation is needed for the selection and/or robust expression of an OR locus;[18] and (3) many and/or all non-expressed OR genes are sequestered within heterochromatic chromocenter compartments in OSN

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nuclei, and disruption of this compartmentalization results in the detection of additional OR RNAs per cell, suggesting that the loss of heterochromatin structure is the rate-limiting step in OR transcription.[19]

Several mechanistic aspects of this model remain unclear, including descriptions of how the repressive H3K9me3 marks are initially laid down at OR loci, how OR gene loci are sequestered within chromocentric compartments, how a single OR locus per cell is selected and liberated and/or precluded from these compartments, and how switching from one OR gene to an alternative OR gene transpires in the event that the initial OR selection is unproductive.[20,21] An obvious version of this model would be that global H3K9 methylation at OR loci drives sequestration within the heterochromatic chromocenters, and a stochastic H3K9 demethylation event per cell permits a single OR locus to escape from this repressive compartment. A problem with this specific hypothesis is the observation that the LSD1 knockout mouse nevertheless results in OSNs expressing a small subset of OR genes, albeit at reduced levels.[18] The fact that ORs are still selected in these mice suggests that either: (1) other demethylases can function to remove the H3K9 mark on the selected OR even in the absence of LSD1, in which case we would need to postulate the coordination of multiple H3K9 demethylases that nevertheless restrict their activity to a single OR locus. Or, (2) removal of an H3K9 mark is not an absolute prerequisite in the selection of every OR gene (e.g., if some OR genes are selectable without demethylation), in which case this specific hypothesis must be discarded, since H3K9 demethylation would not always be the rate-limiting step as predicted.

Alternatively, mutually exclusive OR transcription could be established independent of H3K9 demethylation. For example, the selected OR might be exclusively liberated away from the repressive compartment by a rate-limiting chromatin modifier that operates upstream and/or downstream of LSD1. Or, the selected OR might exclusively access an expression compartment, which precludes it from subsequent sequestration to the chromocenters during commitment. An important distinction between these two hypotheses is the ordering and role of OR sequestration during establishment and maintenance of monogenic OR transcription. The former predicts that monogenic OR transcription cannot be established during development until all competing OR loci are safely sequestered; OR sequestration, in this case, operates as a critical upstream requirement in order to establish monogenic OR transcription. The latter on the other hand, predicts that monogenic OR transcription will precede sequestration; sequestration, in this case, would serve more of a downstream maintenance function during commitment (e.g., to prevent further switching). In this paper, we aimed to distinguish between these two hypotheses by investigating the nuclear organization and transcription of OR genes in cell types derived from earlier stages in the OSN lineage in order to gain insights as to which happens first, OR sequestration (as predicted by the first hypothesis) or monogenic OR transcription (as predicted by the second hypothesis).

We used an immortalized cell type derived from a post-progenitor immature receptor neuron at an earlier stage in the OSN lineage (the OP6 cell line).[22] The OP6 cell line can be induced to further differentiate along the lineage by addition of retinoic acid and by deactivation of the temperature-sensitive *large-T-antigen* permitting exit from the cell cycle.[22,23] Both undifferentiated and differentiated OP6 cells express OR genes monogenically (ref. 23 and herein) and monoallelically (herein), albeit at much lower levels than mature OSNs. Interestingly, OP6 cells frequently switch OR expression during culturing,[23] suggesting that these cells might represent a stage prior to commitment and/or stabilization of OR choice, or alternatively, OR choice has been destabilized by re-entry into the cell cycle when producing the cell line.

Surprisingly, we find that the organization of OR loci in OP6 cells differs significantly from observations made in more mature OSNs. While OR loci are enriched within nuclear chromocenters in OP6 cells, they are commonly found at the nuclear periphery, as well as broadly dispersed in the interchromatin compartments. A given OR locus exhibits diverse positioning within small clonal populations, suggesting that OR-chromocenter interactions in OP6 nuclei might be transient in nature. While the transcribed OR locus is always found external to chromocenters, as observed in mature OSNs,[19] we find that multiple OR loci, including both alleles, are disaggregated and also commonly reside external to chromocenters in each OP6 cell. Thus, unlike in mature OSNs, monogenic and/or monoallelic OR transcription in OP6 cells does not require sequestration of other competing OR loci. OR regulatory mechanisms in OP6 cells could mirror those occurring in immature cell types of the OSN lineage; if so, our results suggest that sequestration of ORs within chromocenters might serve a more downstream function in maintaining OR silencing in mature OSNs as opposed to functioning in the initial establishment of monogenic and/or monoallelic OR transcription earlier in the lineage.

Results and Discussion

Chromocenter organization in OP6 cell nuclei

Nuclear chromocenters are densely packed heterochromatic DNA enriched in H3K9me3 marks and major satellite repeats.[14,24,25] Chromocenters can therefore be visualized by a number of staining methods, including non-uniform TO-PRO-3 iodide staining that displays regions of maximum DNA density, immunofluorescence using antibodies against H3K9me3 histone marks, and direct detection of major satellite DNA by DNA FISH. These independent visualization methods confirm a conventional chromocenter organization in undifferentiated OP6 cell nuclei (**Fig. 1**) that resembles the organization in other cell types.[14] Chromocenters are numerous (approximately 30 per nucleus) and broadly distributed within undifferentiated OP6 cell nuclei (**Fig. 1 and 2**), and there is non-overlap between chromocenters and RNA polymerase II factories (**Fig. 1C**). Therefore, nuclear organization in undifferentiated OP6 cells does not resemble the organization previously observed in mature OSNs, where chromocenters are combined into one or a small number of aggregated foci.[19] Instead, these cells more closely resemble the

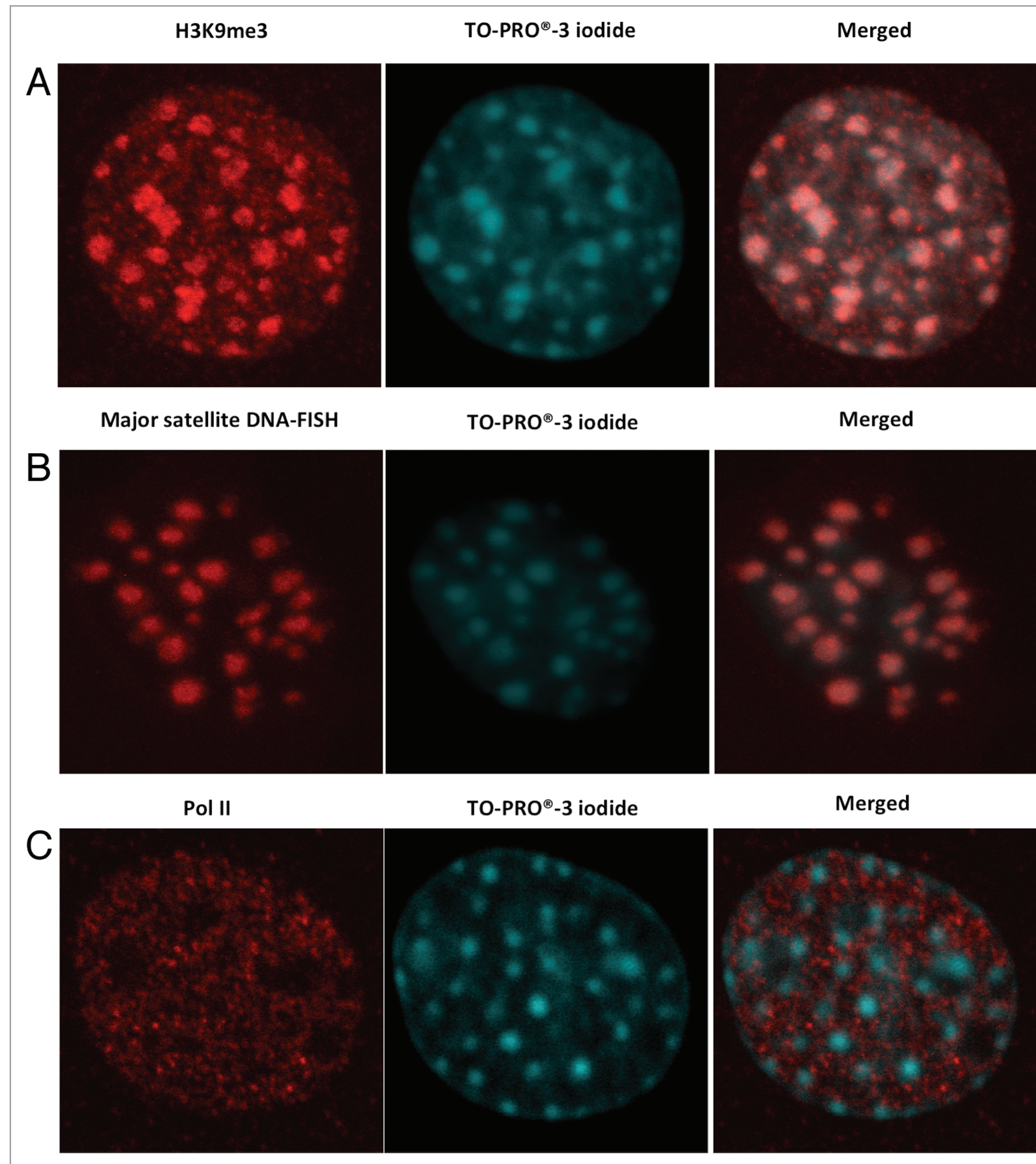

Figure 1. Nuclear chromocenters are marked by DNA density, major satellite DNA, and H3K9 methylation. (**A**) Nuclear chromocenter compartments, as visualized by intense TO-PRO-3 DNA staining (blue) correlates with elevated H3K9me3 immunofluorescence (red). (**B**) Nuclear chromocenter compartments, as visualized by intense TO-PRO-3 DNA staining (blue) correlates with locations of major satellite DNA (red), as visualized by DNA FISH. (**C**) RNA polymerase II factories as visualized by immunofluorescence (red) do not overlap with chromocenter compartments (blue).

organization evident in other mammalian cell types,[14,26] including basal and sustentacular cells of the olfactory epithelium.[19]

We next differentiated OP6 cells by deactivation of the temperature-sensitive *large-T-antigen* and shifting to a differentiation media containing retinoic acid.[22,23] Differentiation under these conditions is slow, asynchronous, and incomplete. After 15 d differentiation, we observe that ~41.6% of the cells develop neurite extensions, including ~23.3% that exhibit a bipolar morphology (**Fig. 2D**). We observe a significant increase in chromocenter aggregation as differentiation progresses (**Fig. 2B-D**). Since we only rarely observe cells with fully aggregated chromocenters, our results suggest that differentiated OP6 cells represent an intermediate state between a dispersed chromocenter organization in undifferentiated OP6 cells and the aggregated chromocenter organization characterized in the most mature cells of the OSN lineage.[19,27]

The progressive transition from a typical chromocenter distribution to a more aggregated chromocenter organization is also observed during the differentiation of rod photoreceptor cells, where there is increasing movement of heterochromatin from the periphery and subsequent aggregation within a diminishing number of chromocenters.[28] The chromocentric aggregation during differentiation of both rod photoreceptor cells and mature OSNs is dependent on the loss of the lamin-B-receptor (LBR),

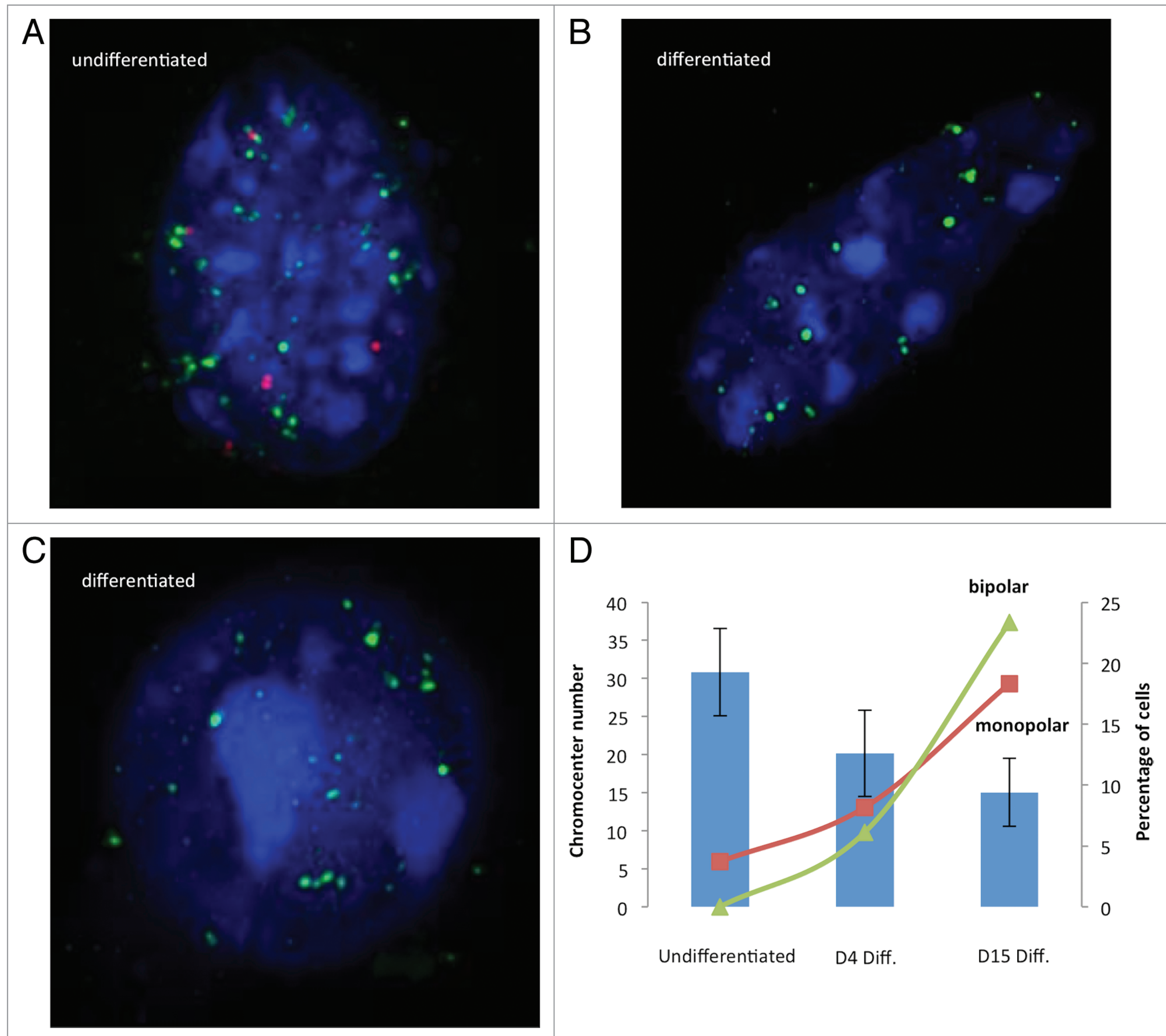

Figure 2. Differentiated OP6 cells exhibit more consolidated chromocenter organization, yet OR genes are not sequestered. (**A-C**) Select images showing typical chromocenter organization (blue) in undifferentiated (**A**) and differentiated OP6 cells (**B**). Pooled DNA FISH probes (RP24–378K9, RP23–275I28, RP23–289G7, RP23–21E22, RP23–359J17, RP23–54M12, RP23–172N22, RP24–65B23) against multiple OR loci are shown (green dots) to illustrate OR distributions relative to chromocenters (also see **Fig. 5B and C** for additional images of pooled probes). (**D**) The average number of chromocenters per nucleus decreases as OP6 differentiation progresses between from day-4 (D4) to day-15 (D15) as shown in the histogram (**D**). Differentiation is incomplete and heterogeneous, with only a minority of cells exhibiting long, bipolar extensions (**D**). Although no examples of complete chromocenter consolidation were observed in OP6, we do see more severe examples of chromocenter consolidation (**C**) when differentiating under similar conditions another OP cell line, OP27.[22,23] The OR genes contained within each BAC probe are indicated in the Methods.

which must be downregulated from the nuclear periphery in order to permit internalization of heterochromatin, which promotes chromocenter fusion.[19,26] With this precedent in mind, we next investigated LBR expression in undifferentiated and differentiated OP6 cells to further elucidate the relationship between LBR expression and chromocenter organization.

Lamin-B-Receptor (LBR) expression in OP6 cell nuclei

The lamin-B-receptor (LBR) is developmentally downregulated during OSN differentiation, leading to the internalization of heterochromatin from the nuclear periphery and consolidation of chromocenters inside the nucleus.[19] When LBR is reactivated in OSNs, the chromocenters revert to a more dispersed organization, suggesting that downregulation of LBR in this lineage is necessary for chromocenter aggregation.[19] In most cell types, LBR is located at the nuclear periphery and functions to maintain a heterochromatic compartment within the nuclear lamina[29,30]; we confirmed the normal LBR distribution at the outer edges of nuclei in GD25 cells, a clonal cell line derived from a fibroblast founder cell that we presumed would have typical LBR distribution in the nucleus (**Fig. 3A**).

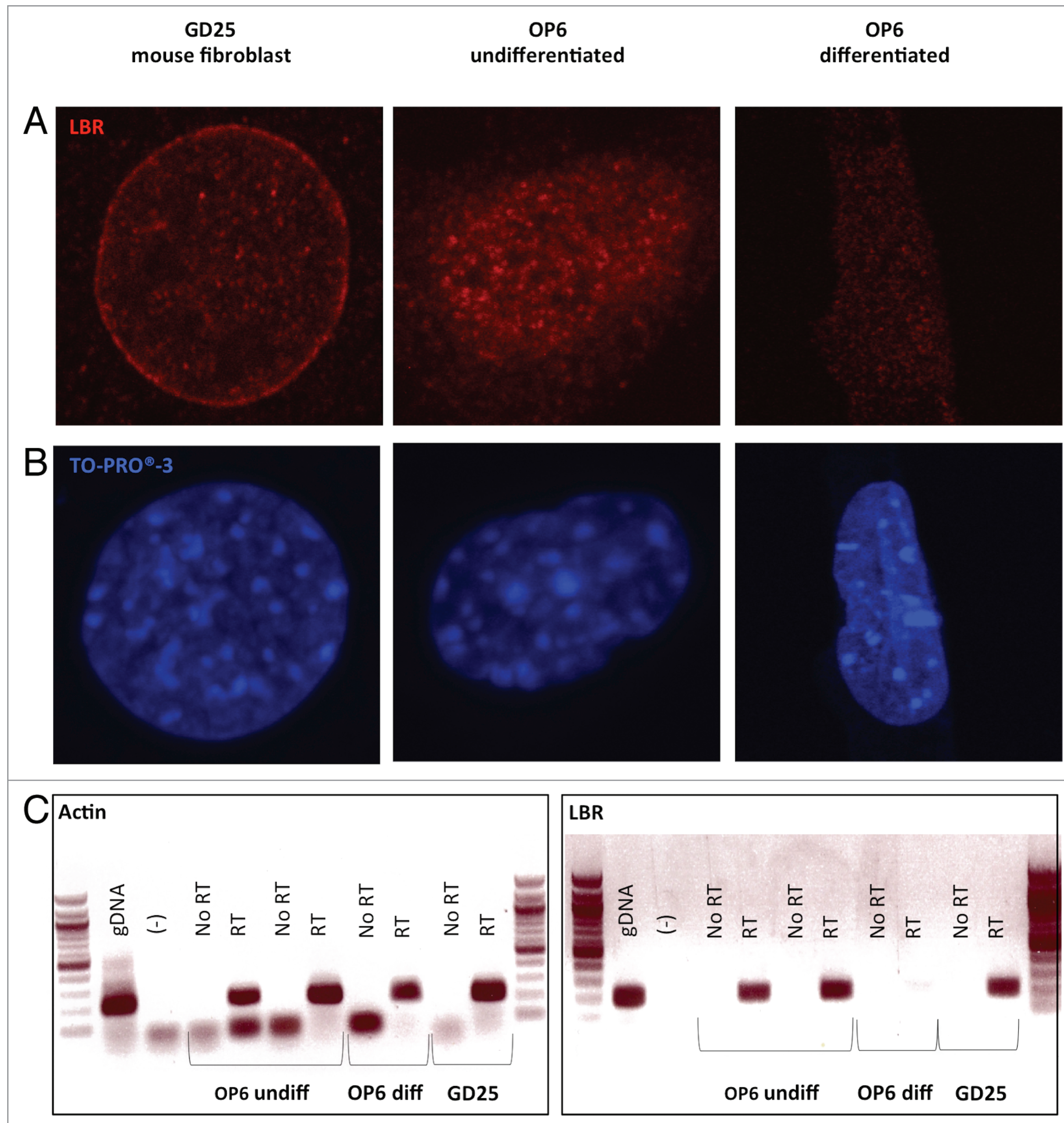

Figure 3. LBR expression in undifferentiated OP6 cells is non-peripheral, and is downregulated during OP6 differentiation. (**A**) LBR immunofluorescence (top panel) for the mouse GD25 fibroblast cell line (left) showing typical staining at the nuclear periphery, as compared with internal LBR localization in undifferentiated OP6 cells (middle) and loss of LBR expression in differentiated OP6 cells (right). (**B**) These same cells are stained with TO-PRO-3 to visualize chromocenters (dense blue). (**C**) Robust LBR expression in GD25 and undifferentiated OP6 cells, as well as LBR downregulation in differentiated OP6 cells, are confirmed by RT-PCR. Actin is used as a control to show approximate equivalency of cDNA preparations between samples.

Interestingly, undifferentiated OP6 cells do not exhibit peripheral LBR staining, and instead LBR protein in these cells is diffusely distributed inside the nucleus (**Fig. 3A**). Thus, undifferentiated OP6 cells appear to exhibit an intermediate state between the common peripheral localization (e.g., in GD25 cells) and the complete downregulation evident in mature OSNs and rod photoreceptor cells.[19,26] LBR internalization might be an important preliminary step to facilitate the internalization of peripheral heterochromatin. We note, however, that chromocenters in undifferentiated OP6 cells remain dispersed (**Fig. 3B**); therefore, LBR relocalization is not sufficient to drive chromocenter aggregation at this stage.

We do not detect LBR expression in differentiated OP6 cells (**Fig. 3A and C**), consistent with a developmental progression toward mature OSNs. As noted (**Fig. 2**), differentiated OP6 cells exhibit partial chromocenter aggregation in the absence of LBR. It seems likely that chromocenter aggregation (and more generally, OSN differentiation) is slow and/or incomplete in these cells due to dependence on other developmental factors that are missing as a consequence of being isolated from normal in vivo environments.

Nuclear organization of OR gene loci in OP6 cells

In mature OSNs, OR genes co-aggregate with consolidated chromocenters,[19] and accordingly, exhibit heterochromatic

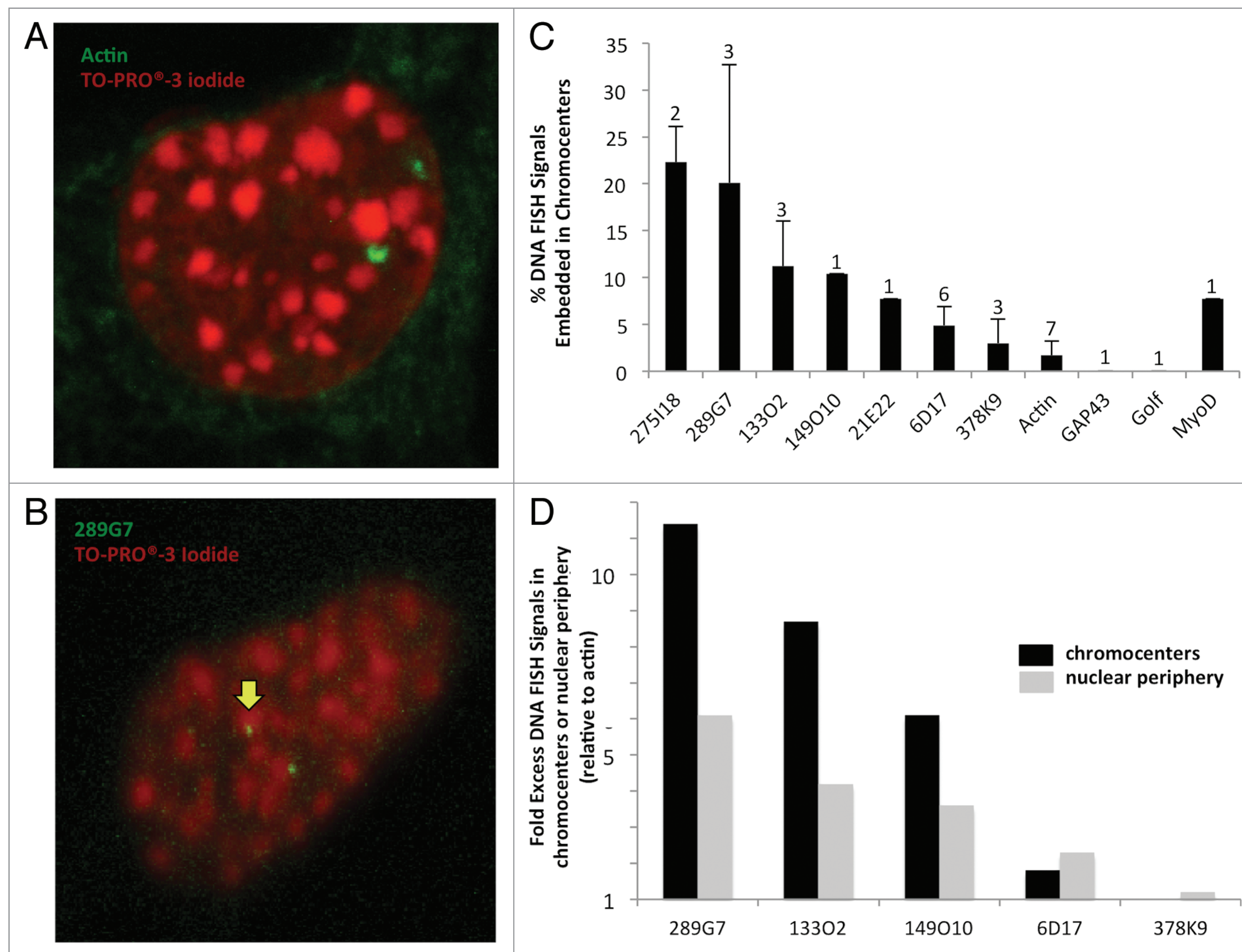

Figure 4. OR loci exhibit variable enrichment within chromocenter and peripheral nuclear compartments. DNA FISH was conducted on undifferentiated OP6 cells using BAC probes containing OR (RP23–275I18, RP23–289G7, RP23–133O2, RP24–149O10, RP23–21E22, RP23–6D17, RP24–378K9) and non-OR(RP23–5J14 [Actin], RP23–454D3 [GAP43], RP23–155O16 [Golf], RP23–358O6 [MyoD1]) genes. Signals were scored from average 50 cells for radial positioning and locations within nuclear chromocenters visualized by TO-PRO-3 staining (red). (**A**) A typical result for BAC RP23–5J14 containing the mouse *actin* locus, where both alleles (green) are positioned in the interchromatin compartment and external to chromocenters (red). (**B**) A common result for BAC RP23–289G7 containing nine OR genes, showing one allele embedded within a chromocenter (yellow arrow) and a second allele at the edge of another chromocenter (green). (**C**) Histogram showing average chromocenter occupancy levels for seven OR-containing BACs tested (left side) as compared with four control loci (right side). Variability in occupancy observed for independent OP6 cell cultures is indicated by standard deviation bars (number of independent experiments indicated above the standard deviation bars). (**D**) Histogram showing the average fold-enrichment in chromocenters and at the nuclear periphery for five OR-containing BACs as compared with the *actin* locus. The OR genes contained within each BAC probe are indicated in the Methods.

marks, such as H3K9me3 and H4K20me3,[17] that are hallmarks of these compartments.[14,24] We next investigated the positioning of several OR loci within nuclei of undifferentiated OP6 cells to ask two questions: (1) Do OR genes associate with chromocenters even in the absence of chromocenter aggregation? (2) Do OR-chromocenter associations and/or disassociations influence OR selection probability and expression status in these less mature cell types?

We conducted DNA FISH experiments using BAC-sized probes encompassing multiple OR genes per probe. We scored four phenotypic categories across OP6 cell populations: the frequency of OR probe signals unambiguously embedded within nuclear chromocenters ("chromocentric"), the frequency of OR probe signals unambiguously external to nuclear chromocenters ("interchromatin"), the frequency of OR probe signals "touching" or immediately peripheral to a chromocenter ("chromocentric-boundary"), and the frequency of OR probe signals "touching" the outer nuclear membrane or located in the outer ~5% of the nuclear radius ("nuclear periphery"). Our results are summarized in **Figure 4**.

As anticipated, a BAC probe containing the *actin* housekeeping gene, as well as a probe containing the active *GAP43* developmental gene were almost exclusively located within the "interchromatin" compartment where RNA polymerase factories

Table 1. Summary of DNA FISH results in OP6 cells using OR-containing BAC probes. Seven OR-containing BAC probes are shown in order of decreasing occupancy within nuclear chromocenters in undifferentiated OP6 cells, as assayed by DNA FISH and TO-PRO-3 visualization of the chromocenters. The table shows chromosomal location (chromosome, approximate megabase position), the number of OR genes contained in the BAC, the overall cluster size, the percentage of LINE1 repeats in the cluster, and the approximate level of "cluster transcriptional activity" (see Methods for definitions)

BAC Probe	Chr.Mb	ORs in BAC	ORs in Cluster	Percent Chromocenter	Percent LINE	Expression Activity
RP23–275I18	Chr9.20	4	6	22.3	43.9	High
RP23–289G7	Chr9.39	9	99	20.1	29.8	Medium
RP23–133O2	Chr10.78	7	8	11.2	55.4	Inactive
RP24–149O10	Chr3.97	1	1	10.4	25.2	Inactive
RP23–21E22	Chr2.86	14	218	7.7	34.3	Medium
RP23–6D17	Chr14.53	4	7	4.9	47.3	Inactive
RP24–378K9	Chr15.98	4	9	3.0	22.8	High

are located (**Fig. 4C**). Surprisingly, we note that two BAC probes containing the *MyoD* and *G-olf* developmental genes, neither of which is expressed in this cell type and is therefore expected to reside in facultative heterochromatin at the nuclear periphery, also tended to be commonly located in the interchromatin compartments (not shown). As discussed previously, the non-peripheral localization of LBR in undifferentiated OP6 cells could indicate that facultative heterochromatin, typically sequestered at the nuclear periphery, is relocated to the inner regions of the nucleus along with LBR. To test this hypothesis, we visualized H3K27me3, a mark of facultative heterochromatin, in OP6 and GD25 cells (**Fig. S1**). We observe enrichment of these marks at the nuclear periphery of GD25 cells, consistent with a more typical peripheral localization of facultative heterochromatin,[12,31-33] but observe a diffuse, internalized localization of H3K27me3 marks in undifferentiated OP6 cells, consistent with the release and inward movement of facultative heterochromatin (as defined by H3K27me3, LBR, and developmentally silenced genes) in this cell type. We note that in differentiated OP6 cells, the facultative heterochromatin, as defined by H3K27me3, remains dispersed in the nuclear interior; we see no evidence of H3K27me3 consolidation around chromocenters as was observed in mature OSNs in vivo.[27] Thus, the chromatin organization in OP6 nuclei appears to be an intermediate state between a typical non-OSN cell (with peripheral LBR and peripheral facultative heterochromatin, and containing several distinct constitutive heterochromatic chromocenters) and a fully differentiated OSN (lacking LBR and consolidated facultative and constitutive heterochromatin).

As compared with the control probes, we found that OR loci exhibit a significantly higher frequency of "chromocentric," and "nuclear periphery" localization in undifferentiated OP6 cells (**Fig. 4C and D**), consistent with the previous observation that OR gene loci are decorated with heterochromatic marks, such as H3K9me3 and H4K20me3.[17] The remaining OR gene loci located in non-peripheral and non-chromocentric regions of the nucleus appear to be randomly distributed and not biased for proximity to H3K9me3- or H3K27me3-rich compartments. Also, we do not observe consistent patterning for the two OR alleles per cell: in some cells, both alleles are disassociated with chromocenters, and in other cells, one or both alleles are associated with chromocenters. This result contrasts recent observations made in mature OSNs, where the two homologs tend to exhibit a complementary pattern per cell, with one OR allele associated and the other OR allele disassociated from chromocenters.[27]

Chromocenter association for a given OR BAC probe is not consistent from cell to cell, even among small cell clones with recent common ancestry. Given that the OP6 cell line is clonal, the heterogeneous distribution of a given OR probe from cell to cell suggests that OR allelic interactions with chromocenters are transient in these dividing cells; e.g., associations might be stabilized and/or destabilized at various points in the cell cycle.

Since the frequency of chromocenter occupancy is reproducibly greater with some OR-containing BACs (e.g., *275I18*) vs. others (e.g., *6D17*), we wondered if these tendencies correlated with either the repetitive sequence properties of a locus or the expression status of the OR genes at a locus. We find no evidence to support either hypothesis. Neither the repeat content (e.g., LINE repeats) nor the number of tandem ORs present in the cluster (cluster size) is predictive of chromocenter occupancy (**Table 1**). For example, BAC probes containing an isolated, unclustered OR gene (e.g., *149O10*) or probes with relatively low LINE repeat content (e.g., *289G7*) are at least as likely to associate with chromocenters as probes from very large OR clusters (e.g., *21E22*) or those densely populated with LINE repeats (e.g., *6D17*).

We also do not find correlations between the chromocenter occupancy and the observed transcriptional activity of OR genes encompassed by the probe (**Table 1**). For example, two of our probes contain OR genes expressed in >3% of OP6 cells and were classified as "highly active OR clusters" (see Methods), one of which exhibits very high occupancy levels (275I18, ~22%) and the other very low occupancy levels (378K9, ~3%). Moreover, the *6D17* probe containing "inactive" OR genes (i.e., encompassing ORs never detected in OP6 populations, even with exhaustive searches via tiling arrays; not shown) exhibits one of the lowest incidences of chromocenter associations (<5%), indicating that the silencing of this locus is accomplished by some other means. Together, our results suggest that chromocenter occupancy is unlikely to play a dominant role in the regulation of OR locus selection probability during OR switching in OP6 populations.

OR genes are not sequestered within OP6 cell nuclei

In mature OSNs, disruption of chromocenter consolidation by LBR upregulation results in the disassociation of OR

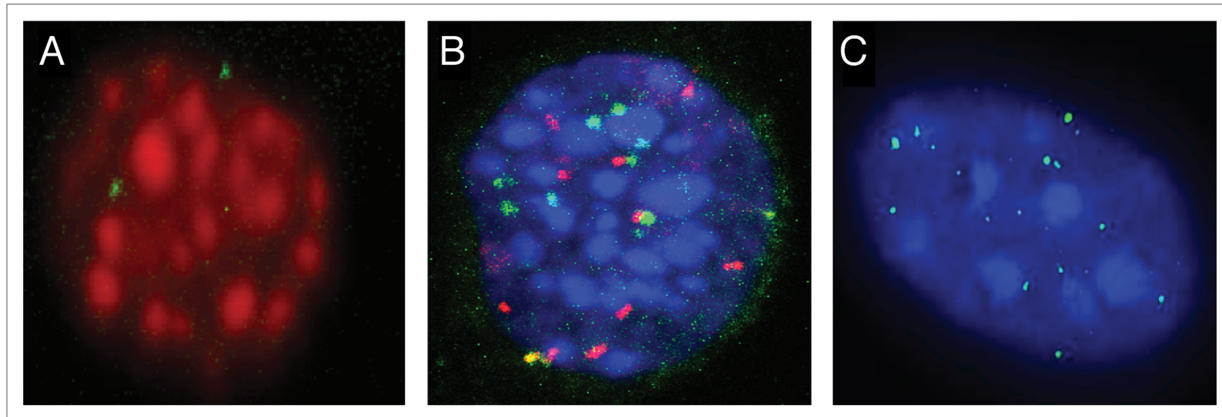

Figure 5. Multiple OR genes and/or alleles are dispersed in the interchromatin compartment in undifferentiated and differentiated OP6 cells. DNA FISH using OR-containing BAC probes on OP6 cell nuclei stained with TO-PRO-3 to visualize nuclear chromocenters. (**A**) DNA FISH conducted with a single OR-containing probe (RP23–275I18) showing a common result where both alleles (green) are positioned external to nuclear chromocenters (red). (**B**) DNA FISH conducted with six OR-containing probes (two pools each with three probes, red [RP23–133O2, RP24–149O10, RP23–6D17] and green [RP23–289G7, RP24–378K9, RP23–275I18]), showing a common result in undifferentiated OP6 cells where multiple OR gene loci are dispersed within the interchromatin compartment and external to chromocenters (deep blue). (**C**) DNA FISH conducted with a pool of four OR-containing probes (green) (RP24–378K9, RP23–275I18, RP23–289G7, RP23–21E22), showing that OR loci are not sequestered within nuclei of differentiated OP6 cells. The OR genes contained within each BAC probe are indicated in the Methods.

genes and inappropriate expression of multiple OR genes per cell.[19] Therefore, it appears that in mature OSNs, the only barrier to preserve mutually exclusive OR expression is the sequestration of competing ORs within an internalized heterochromatic compartment. Although our DNA FISH data suggests a variable nuclear location for a given probe from cell to cell (periphery, interchromatin, or chromocenter), it remains possible that OR gene loci nevertheless aggregate together (independent of location) in order to preserve monogenic and monoallelic OR expression.

To test this hypothesis, we conducted DNA FISH with pooled BAC probes from multiple chromosomal locations. We find no evidence for significant OR aggregation of OR loci, either in association with chromocenters or within the interchromatin compartments (**Fig. 2A and 5B**). Therefore, unlike in mature OSNs, OR loci in undifferentiated OP6 cells are not sequestered; e.g., within one or a small number of heterochromatin compartments.

Since chromocenter and OR aggregation depends on LBR downregulation in mature OSNs, we next used our pooled OR probe in differentiated OP6 cells, where LBR expression is downregulated and chromocenters show signs of consolidation. We still do not see evidence for OR sequestration after 15 d of differentiation (**Fig. 2B and C and 5C**). These results suggest that OR sequestration is not merely a consequence of LBR downregulation or chromocenter restructuring, but that some additional developmental condition is necessary but presumably missing in these partially differentiated OP6 cells.

OR sequestration is not required for monogenic and/or monoallelic OR expression in OP6 cells

As referenced previously, the mutually exclusive transcription of OR alleles in mature OSNs is dependent on the sequestration of competing gene loci to a consolidated chromocentric compartment.[19] The fact that OP6 cells do not exhibit an aggregated chromocenter compartment (**Fig. 2**), and OR gene loci are not sequestered to one or a small number of heterochromatic locations (**Fig. 2 and 5**), challenges the conclusion that OR sequestration is a necessary and sufficient explanation to account for the establishment of monogenic/monoallelic OR transcription in these olfactory placode-derived cells from the OSN lineage.

We had previously sequenced degenerate PCR products produced from single OP6 cells to demonstrate that these cells express one and only one OR gene per cell.[23] We extended those analyses here in three ways: (1) we performed single-cell degenerate PCR sequence analyses on additional OP6 cells, not just to increase sample size but also to ensure that this important property of the cell line has not changed since the original analyses; (2) we performed single-cell degenerate PCR restriction digest analyses on single OP6 cells to mitigate against potential sampling biases inherent in the sequencing approach; and (3) we performed RNA FISH on OP6 cells to demonstrate monoallelic OR expression, as well as to correlate percentages of positive cells for a given OR gene with estimated overall OR diversity in the population at large. All of our data are consistent with our previous conclusion that OR alleles and/or genes are expressed in a mutually exclusive way in individual OP6 cells (**Fig. 6**; **Table 2**). We elaborate on these findings below.

We isolated RNA from three single undifferentiated OP6 cells, and used degenerate PCR primers designed against well-conserved sequences encoding transmembrane domains 2, 3, 6, and 7 of mouse OR genes.[1,4,19] We cloned and sequenced several individual templates from these PCR reactions, and found only one dominant OR present per cell (**Table 2**). We note that with increased sample sizes, we begin to identify additional isolated OR templates along with the dominant OR, but we argue that this is noise likely arising from low levels of genomic DNA (gDNA) contamination for the following reasons. First, follow-up PCR reactions using primers designed specifically against ORs

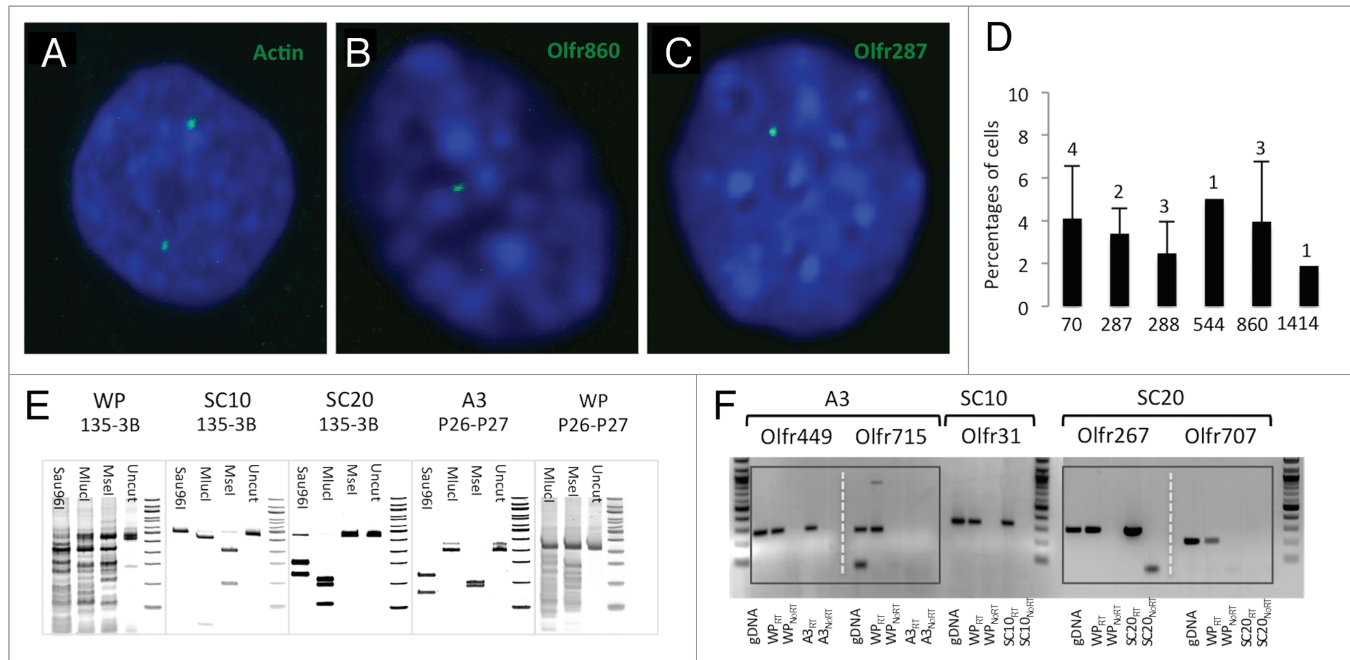

Figure 6. OP6 cells express OR genes monoallelically and monogenically. (**A-C**) RNA FISH conducted on OP6 cells showing a typical two-spot signal for an actin-specific probe (**A**), and a typical one-spot signal for two OR-specific probes (**B-C**). (**D**) Histogram summarizing percent positive cells in multiple independent RNA FISH experiments (number of experiments per probe shown above standard deviation bars) for six abundantly expressed OR genes (Olfrs 70, 287, 288, 544, 860, and 1414). Each experiment scored at least 500 OP6 cells. (**E**) Degenerate RT-PCR products (using either the 135–3B or the P26-P27 degenerate PCR primers) generated from the SC10, SC20, and A3 single cell cDNAs are homogeneous as evident by restriction digest analyses and sequencing (see **Table 2**), as compared with heterogeneous composition in products generated from whole OP6 populations (WP). Digestion products match expected sizes for *Olfr31* in SC10, *Olfr 267* in SC20, and *Olfr31* in A3. These data extend additional single-cell samples previously published in Pathak et al.[23] (**F**) Gene-specific PCR confirms the robust presence of the one dominant OR detected in each single cell (*449* in A3, *31* in SC10, *267* in SC20) by sequencing; isolated, spurious OR templates identified in degenerate PCR products are not evident by direct gene-specific PCR amplification (*715* in A3, *707* in SC20). All gene-specific PCR products were generated on genomic DNA (gDNA), as well as cDNA isolated from whole cell populations (WP); no products are detected in no-RT controls.

Table 2. Summary of sequence results from OR degenerate RT-PCR conducted on OP6 single cells. The number of sequenced templates confidently scored (after discarding poor quality sequence reads) with >98% identity to a specific OR gene in the latest mouse genome assemblies for each sampled degenerate RT-PCR product is indicated. Also indicated is the dominant OR identified, the number of independent templates matching the dominant OR ("hits"), and isolated secondary ORs, if any, comprising the rest of the sample. See **Figure 6** for additional characterization of these degenerate OR products

Clone ID	Sequenced Templates	Dominant OR	Hits	SecondaryORs
OP6-A3	26	Olfr449	24	Olfr1335, Olfr715
OP6-SC20	27	Olfr267	25	Olfr1329, Olfr707
OP6-SC10	5	Olfr31	5	none

identified by sequencing, results in a strong positive product for the dominant gene but no visible products for other spurious OR templates identified in the original degenerate reaction (**Fig. 6F**). Second, we do not find multiple samples of any specific OR, except for the dominant OR, in any given cell (**Table 2**). We note that gDNA has as many as a thousand viable OR templates per molecule (i.e., there are >1000 OR genes encoded in the genome), so a small, random sampling from a gDNA contaminant is unlikely to contain redundancies. In contrast, if there was a small number of transcriptionally active OR loci per cell, we would probably detect multiple RNA templates of each OR present given this sample size. And third, we digested these degenerate PCR products with frequent-cutting restriction enzymes and identify a single digestion product (**Fig. 6E**), consistent with criteria used by others to argue that OSNs express only one OR gene per cell.[4,19,34] The degenerate-PCR and restriction-digest approach in these experiments is sufficiently sensitive to detect multiple ORs in RNA populations when they exist, as evident in controls with preparations from whole populations (**Fig. 6E**). However, we caution that this widely used method for asserting monogenic OR expression is limited by a false-negative rate on single-cell cDNA preparations that we estimate to be between 30–70%, depending on the degenerate PCR primers used. Although we have never observed more than one OR product in these and in previous single-cell analyses,[23] and we routinely combine three separate PCR reactions from the same cDNA preparation prior to

digestion in order to decrease the false-negative probability, there remains the possibility that additional OR cDNAs are present but not detected.

We additionally conducted RNA FISH on OP6 populations using several OR gene-specific probes. We make three important observations from these experiments (**Fig. 6**). First, positive hybridization signals are always located within the interchromatin compartment. This result is consistent with observations made previously in mature OSNs, where the actively transcribed OR locus is separated from the chromocenter compartment, presumably so that it can access an RNA polymerase II factory. Second, we identify exactly one RNA FISH signal per positive cell for each probe tested. This result confirms that OP6 cells express OR genes monoallelically. Third, we scored the percentage of positive cells for RNA FISH for a panel of six ORs independently characterized in tiling arrays as among the top 25% of ORs represented in the undifferentiated OP6 population (tiling array data not shown; manuscript in preparation). According to tiling array data, we found that undifferentiated OP6 populations express ~80 OR genes. Therefore, assuming monogenic expression, we would estimate an average frequency of approximately 1.25% per gene. Assuming a Poisson distribution with an average density of 1.25%, we would predict that the top ~25% of the distribution would fall between 3–5% per gene, consistent with our observations (**Fig. 6D**).

Together, our data argue that OP6 cells express one and only one OR allele per cell by criteria used by others in support of this same conclusion in mature OSNs.[4,19,34] We have shown that in a given OP6 cell, OR gene loci are dispersed throughout the interchromatin compartment, presumably with hundreds of OR genes located outside of chromocenters in each cell. We also observe that the two OR alleles both commonly reside outside of chromocenters, noting that RNA FISH conducted with gene-specific probes from these loci never reports more than one actively transcribed allele per cell. Therefore, we propose that sequestration of OR genes/alleles into one or a small number of confined heterochromatic compartments is not a requirement for monogenic and/or monoallelic OR expression in OP6 cells, which contrasts conclusions drawn for mature OSNs.[19] We discuss possible ways to reconcile these two apparently contradictory conclusions in the following section.

Do OP6 cells represent a pre-committed state along the lineage?

In this paper, we show that OP6 cells exhibit a nuclear organization phenotype that falls between the basal cells of the OSN lineage (where LBR is expressed at the nuclear lamina, chromocenters are dispersed, and OR genes are transcriptionally inactive) and mature OSNs (where LBR is not expressed, chromocenters are consolidated, one OR is robustly expressed, and all other transcriptionally inactive OR genes are sequestered within the large, internal heterochromatic compartment). In OP6 cells, OR gene loci are not sequestered (**Fig. 2 and 5**), and only after inducing further differentiation of these cells, do we observe progression to the more mature(-like) state where LBR is downregulated (**Fig. 3**) and chromocenters begin to consolidate (**Fig. 2**).

OP6 cells exhibit three other properties that seem to suggest they represent an intermediate developmental state. First, although OR transcription appears to be monogenic and/or monoallelic, these cells express OR genes at a much lower level per cell than a mature OSN (not shown), where in the latter the expressed OR represents one of the most abundant transcripts in the cell.[35] Second, OP6 cells frequently switch their OR choice during growth of clonal colonies (manuscript in preparation), suggesting that these cells represent a pre-committed and/or unstable state. Third, we previously reported that OR mRNA in undifferentiated OP6 cells is retained in the nucleus.[23] Therefore, the combination of low transcriptional levels and low and/or no OR protein translation (because OR mRNA is not efficiently exported from the nucleus) may fail to trigger the ER-stress response and subsequent receptor-mediated feedback loops shown to be critical for stabilizing OR choice, as well as increasing OR expression levels during OSN maturation.[20,21,36]

Taken together, we speculate that OP6 cells represent a stalled, pre-committed state along the OSN maturation process. If full OSN maturation includes a positive feedback loop that significantly increases OR expression level as part of the commitment process, as has been described,[36] then this feedback presumably includes the upregulation of a potent transcription factor and/or chromatin-modifying complex. Perhaps only in the presence of this putative enhancer complex in committed OSNs is there a new requirement for OR sequestration in order to maintain monogenic OR expression. This hypothesis predicts that rare, pre-committed cells in vivo would exhibit similar "intermediate" characteristics as OP6 cells: internalized LBR and H3K27me3, non-consolidated chromocenters and OR loci, and monogenic/monoallelic OR expression at low levels. We are currently investigating this question in the earliest post-mitotic cells of the developing mouse OSN lineage.

Methods

Cell cultures

The OP6 cell line was cultured under media conditions described previously.[22,23] Briefly, OP6 cells are grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Gibco) at 33 °C and differentiated by deactivating the large-*T-antigen* at 39 °C for 4–15 d in DMEM-F12 media (Life Technologies) containing N2 supplement (Life Technologies), 100 μM ascorbic acid (Sigma), and 10 μM retinoic acid (Sigma). GD25 cells were maintained in DMEM-high glucose (Life Technologies) with 10% FBS (Gibco) containing 2 mM L-glutamine (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). For subsequent immunofluorescence and FISH analysis, cells were seeded on 22 cm^2 coverslips coated with 0.1% gelatin (Sigma) in a 6 well plate at about 50% confluency and expanded for one day to near confluency.

Immunofluorescence

Immunofluorescence experiments were modified slightly from procedures described elsewhere.[37] Briefly, OP6 and GD25 cells were fixed with 3% paraformaldehyde for 10 min, permeabilized

in 0.5% Triton-X (Sigma) for 10 min, and blocked in 1% BSA for 20 min at 37 °C. The primary and secondary antibody incubations were performed at 37 °C for 45 min in a humidified chamber. The primary antibodies used in this study were anti-H3K9me3 (Millipore, 07-442; 1:100), anti-Pol II (Santa Cruz Biotechnology, sc-899; 1:200), anti-LBR (Abcam, ab122919; 1:50), and anti-H3K27me3 (Millipore, 07-449; 1:500). The secondary antibody used was goat anti-rabbit-Cy3 (Millipore, AP132C; 1:800). DNA was stained using TO-PRO-3-iodide (Life Technologies; 1:1000) or DAPI (Sigma Aldrich; 1:1000).

Microscopy and image processing was performed using a Deltavision RT imaging system (Applied Precision) adapted to an Olympus (IX71) microscope and a Zeiss LSM510 confocal microscope. Each image was sectioned with 0.5-micron intervals to ensure complete coverage of the nucleus. Visualization of nuclear chromocenters was accomplished by three independent measures that were equally effective and redundant: regions of maximum major satellite DNA, regions of maximum H3K9me3 staining, and regions exhibiting maximum DNA staining density. Chromocenter counts per nucleus were made by examining each z-section by eye, and marking counted chromocenters in the first z-section they appear to prevent double-counting in subsequent sections.

DNA FISH

DNA FISH was performed using BAC clones obtained from BACPAC Resource Center (CHORI). BAC DNA was nick-translated with DIG or biotin according to manufacturer's instructions (Roche). Approximately 100 ng nick translated probe was mixed with 5μg Cot1-DNA (Invitrogen) and 10mg salmon sperm DNA (Sigma) per reaction. Following cell fixation and permeabilization, cells were dehydrated in an 80%, 95%, 100% ethanol series, prior to incubation in 50% formamide/2X SSC for 20 min at room temperature. Cells were denatured at 85 °C for 30 min and then hybridized with heat-denatured probes overnight at 37 °C. Following hybridization, cells were washed three times with 50% formamide/2X SSC for 5 min each and blocked in 4% BSA/4X SSC/0.2% Tween-20 for 20 min at 37 °C in a humidified chamber. Anti-DIG or avidin antibody incubations in 1%BSA/4X SSC/0.2% Tween-20 were performed for 45 min at 37 °C in a humidified chamber. FISH signals were detected with sheep anti-DIG-FITC (Roche, 11207741910), donkey anti-sheep-FITC (Santa Cruz Biotechnology, sc-2476) for DIG labeled probes, Avidin-FITC (Vector Labs, A-2011) or avidin-rhodamine (Vector Labs, A-2012), and biotinylated anti-avidin (Vector Labs, BA-0300) for biotin labeled probes in various experiments.

The following BAC clones were used as probes in various studies: RP24-273E7 (major satellite repeats), RP23-5J4 (actin), RP23-155O16 (G-olf), RP23-454D3 (GAP43), RP23-358O6 (MyoD), RP23–275I18 (Olfr857, Olfr58, Olfr859, Olfr860), RP23-289G7 (Olfr917, Olfr918, Olfr919, Olfr920, Olfr921, Olfr922, Olfr923, Olfr924, Olfr26), RP23-133O2 (Olfr1356, Olfr1355, Olfr1354, Olfr8, Olfr1353, Olfr1352, Olfr1352, Olfr57), RP24-149O10 (Olfr1402), RP23-21E22 (Olfr1008, Olfr1009, Olfr1010, Olfr1012, Olfr1013, Olfr1014, Olfr1015, Olfr1016, Olfr1018, Olfr1019, Olfr1020, Olfr1022, Olfr1023, Olfr1024), RP23-6D17 (Olfr1510, Olfr1509, Olfr1508, Olfr1507), and RP24-378K9 (Olfr288, Olfr287, Olfr286, Olfr257), RP23-359J17 (Olfr675, Olfr676, Olfr677, Olfr678, Olfr679, Olfr681, Olfr683, Olfr684, Olfr685, Olfr686), RP23-54M12 (Olfr160, Olfr151, Olfr874, Olfr875, Olfr876, Olfr877, Olfr145, Olfr878), RP23-172N22 (Olfr342, Olfr344, Olfr345, Olfr346), RP24-65B23 (Olfr458, Olfr457, Olfr456, Olfr455). We selected OR-containing BACs representative of various levels of OR expression activity in OP6 populations, as determined by cDNA tiling array experiments (not shown; manuscript in preparation). Two BACs (RP23-275I18 and RP24-378K9) were classified as "highly active clusters" because there are multiple, tightly-linked ORs within the top 25th percentile in overall expression level in OP6 populations. Two BACs (RP23-289G7 and RP23-21E22) were classified as "medium active clusters," which either contain one or a small number of active ORs that are each within the top 25th percentile in overall expression level, or contain several, less robustly-expressed ORs. Three BACs (RP23-133O2, RP24-149O10, and RP23-6D17) were classified as "inactive clusters," where we find no evidence for expression of nearby ORs in OP6 undifferentiated populations.

We note that OP6 cell populations exhibit varying degrees of polyploidy at these higher passage numbers (P13 for most studies), commonly resulting in more than two DNA FISH signals per cell. We confirmed that additional FISH signals were due to polyploidy, as opposed to false-positive cross-hybridization, by differentially labeling linked BAC probes encompassing unique regions flanking two of our DNA FISH probes: BACs RP23-63K14 and RP23-22OF2 were used to confirm polyploidy of the OR locus contained within RP24-378K9 on chromosome 15, and BACs RP23-473H10 and RP24-248K12 were used to confirm polyploidy of the *actin* locus contained within RP23-5J14 on chromosome 5.

RNA FISH

Cells were prepared as described for DNA FISH, with the following differences: all reagents and procedures were conducted in RNase-free conditions, permeabilization included CSK buffer with 2 mM vanadyl ribonucleoside complex (VRC, NEB) and the denaturation step required to expose DNA templates in DNA FISH was excluded in RNA FISH. Probes were generated by PCR amplification of specific OR introns and incorporating biotin-16-dUTP (Roche) into PCR products. Each product was fragmented to <500 bp by nick translation (Roche). Approximately 50–100 ng of probe was mixed with 5 μg Cot1-DNA (Invitrogen) and 10mg salmon sperm DNA (Sigma) and heat-denatured. Following an overnight hybridization, cells were washed, blocked, and visualized as described for DNA FISH. The following PCR primers were used to produce long, intronic PCR products: Olfr70_F (GGAGCATTCA TCTGGCATGT) and Olfr70_R (AGGAGAGAAC GCAGCACATT), Olfr287_F (CCACACAGGG ATCTGTAGCA) and Olfr287_R (TTGCAATGTG ATGCTTGGAT), Olfr288_F (TCCCTTCTGC TAGGAAGCTG) and Olfr288_R (CACTTGCCTT TCCGGTTGTA), Olfr860_F (GAGAGACAGG ATTATTACAT TGTTGG) and Olfr860_R (AAACCTTGGT GATGAACTAA GCA), Olfr1414_F

(GCTCTCAGCA TCCTGTCTCC) and Olfr1414_R (AGCCAGCAGG AAGAACAGAC). We confirmed the loss of signal in negative control experiments that included Ribonuclease A (Thermo Scientific). We conducted RNA FISH on six of the most abundantly-expressed OR genes in undifferentiated OP6 populations (**Fig. 6D**), as defined by signal intensity in cDNA tiling array data not shown (manuscript in preparation).

cDNA analyses

For undifferentiated and differentiated OP6 populations, as well as for GD25 cell populations, RNA was harvested using Trizol (Life Technologies), and cDNA produced with equal amounts of RNA from each sample using the SMARTer™ PCR cDNA synthesis kit and 21 amplification cycles (Clontech). The following PCR primers were used to amplify LBR and actin templates using 25 amplification cycles: LBR_F (AAAAAGTGGC TCGATTTCCA) and LBR_R (GCGGAGTCAG CTTAACTTGC), Actin_F (ATCTTCATGA GGTAGTCTGT CAGG) and Actin_R (CATGTTTGAG ACCTTCAACA CCC). For single-cell cDNA studies, we isolated individual OP6 cells by serial dilution into 96-well plates, and confirmed wells containing single cells by microscopy. Verified single cells were lysed in 24 μL 0.05% NP-40 for one minute at 65 °C and one minute and room temperature. Lysates were distributed evenly for RT and no-RT experiments. First-strand cDNA synthesis was performed using oligo-dT 3′ linker primer (AAGCAGTGGT ATCAACGCAG ACTT T_{21}; (modified from ref. 38) and a 5′ linker primer (AAGCAGTGGT ATCAACGCAG AGTAGCAGGG (modified from ref. 38) using SuperScript III reverse transcriptase (Invitrogen) for 90 min at 42 °C. Second-strand cDNA synthesis was performed with Advantage 2 Polymerase (Clontech) using the linker primer (AAGCAGTGGTA TCAACGCAGA GT) and PCR amplifying for 40 cycles at 65 °C annealing temperature. Successful cDNA production and low gDNA contamination was verified by *actin* PCR on RT and no-RT samples, respectively.

Degenerate OR PCR on single-cell cDNA preparations was performed using either the P26 (GCITAYGAYC GITAYGTIGC IATITG)–P27 (ACIACIGAIA GRTGIGAISC RCAIGT) primer pairs, or the 135 (ATGGCITAYG AYMGITAYGT IGCIATHTG)–3B (AGRCWRTAIA TGAAIGGRTT CAICAT) primer pairs for 35 additional cycles at 45 °C annealing temperature. For sequencing, degenerate PCR products were cloned into the TOPO 4.0 vector (Invitrogen) and plasmid DNA was prepared from insert-positive clones. For restriction digests, three independent degenerate PCR products from each cDNA were pooled, gel-purified, and digested with one of four frequent-cutting restriction enzymes (MluCI, MseI, or Sau96I; NEB); banding patterns were visualized on 10% native PAGE gels stained with SYBR Gold (Life Technologies).

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/nucleus/article/29343

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