Immortalisation, Characterisation And Differentiation Of Temperature Sensitive Cell Lines From The Olfactory Neuroepithelium

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Declaration

I hereby declare that this thesis entitled:

Immortalisation, Characterisation And Differentiation Of Temperature Sensitive Cell Lines From The Olfactory Neuroepithelium

is my own work, and has not previously in its entirety or in part been submitted at any university for another degree.

Signed: Signed by candidate

Signature Removed

Date: 31 August 1998

The greatest gift is passion for reading. It is cheap, it consoles, it distracts, it excites, it gives you knowledge of the world and experience of a wide kind. It is a moral illumination.

Elizabeth Hardwick

Dedications

This thesis is dedicated to my parents Ismail and Gadija Boolay, my Aunt Gadija Isaacs, my husband Abdul Kader who patiently supported and encouraged me to achieve this important milestone in my career and my late Uncle Arafiem Isaacs who was my initial encouragement to attend university but was not able to see me complete this degree.

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Immortalisation, Characterisation And Differentiation Of Temperature Sensitive Cell Lines From The Olfactory Neuroepithelium

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Embryonic olfactory neuroepithelium provides a useful experimental system for the study of olfactory neurogenesis.

As a substrate for experimental neural cell biology, olfactory neuroepithelium is of unique interest since, unlike other neural cells, olfactory neurons are continually replaced - a feature that is dictated by their direct exposure to the damaging external environment. Basal cells in the olfactory placode are the source of this replacement.

Each olfactory neuron expresses only one or a few of the many olfactory receptors that are encoded by the large array of olfactory genes. Despite this limited cellular display of receptors, vertebrates are able to distinguish many thousands of different odorants, implying a complicated need for perceptive neurological processing of signals coming from individual olfactory neurons.

To study the events that take place during the differentiation of neuronal precursors - a process that sustains a diverse receptor repertoire - I felt that lines of conditionally immortalised cells that could be induced to differentiate would provide useful reagents.

In this thesis I describe my successful attempts to immortalise olfactory cell lines from the neuroepithelium of E10.5 mouse embryos. I used a conditionally immortalising retrovirus that included the coding sequence for the temperature-sensitive SV40 large T antigen. Integration of this retrovirus into the genome of cells allowed continuous proliferation at the permissive temperature of 33°C. A shift to the non-permissive temperature of 39°C inactivated the SV40 large T antigen, the cells ceased proliferation and differentiation commenced.

Sixty cell lines were derived of which four were chosen for further characterisation. These four cell lines (OP6, OP27, OP47 and OP55) were clonally derived and were immortalised rather than transformed. They continued to express the SV40 large T antigen at 33°C but lost expression at 39°C concomitant with cessation of proliferation.

When the OP cells were shifted to 39°C in the absence or presence of the morphogen, retinoic acid, morphological changes ensued that were consistent with the development of neuronal characteristics. The OP6, OP27 and the OP47 cells became phase-bright with neuritic extensions. The OP55 cells were the exception in that they did not develop extensions but instead differentiated to form compact epithelial islands when grown in DM-10 medium but not in RA medium.

Differentiation of the OP cells at 39°C was further documented by the induced expression of a number of markers demonstrated by RT-PCR and/or immunocytochemistry.

The OP cells differentiated at 39°C in DM-10 and in retinoic acid-containing medium to express olfactory receptor transcripts. Cloning and sequencing showed that each cell line expressed a single receptor type but that different receptors were expressed by different cell lines. Sequencing revealed that the receptors cloned from the OP27 cells were 98% homologous to the mouse-M65 olfactory receptor whereas OP55 had greatest homology to rat-Olf3 olfactory receptor. The transcripts induced in OP6 and the OP47 cells showed greatest homology with Gus58 - a taste receptor homologous to olfactory receptors.

Sequences obtained from OP6, OP47 and OP55 cells were not 100% identical to published receptors and could thus represent members of different subfamilies. Interestingly, induced OP55 cells also expressed mRNA for clusterin - a molecule that has no homology with olfactory receptor transcripts but is involved in differentiation during embryogenesis.

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Abbreviations

AC	adenylate cyclase
ATCC	American type culture collection
ATP	adenosine triphosphate
BDNF	Brain derived neurotrophic factor
BF-1	Brain factor-1
bFGF	basic fibroblast growth factor
bHLH	basic helix loop helix
bp	base pair(s)
BrdU	5-Bromo-2'-deoxy uridine
BSA	bovine serum albumin
cAMP	cyclic 3', 5'-monophosphate
cdk	cyclin dependent kinases
cDNA	complementary DNA
CMF-HBSS	calcium and magnesium free hanks balanced salt solution
CNS	central nervous system
CRABP	cellular retinoic acid binding proteins
CRBP	cellular retinol binding proteins
dNTP	deoxynucleotide triphosphate
DAB	diamino benzidine
DMEM	Dulbecco's Modified Eagles Medium
DM-10	DMEM containing 10% FCS
E.coli	*Escherichia coli*
E10	embryonic day 10
EBF	Early B-cell factor
EDTA	ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
F-12	Ham's F-12 medium
FCS	Fetal calf serum
GBC	globose basal cells
GCG	Genetic Computer Group
gDNA	genomic DNA
GFAP	Glial fibrillary acidic protein
GnRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptors
G-proteins	guanine nucleotide binding proteins
Gus	gustatory
HBC	horizontal basal cell(s)
HNF-3	Hepatocyte nuclear factor-3
H_2O_2	hydrogen peroxide
Hox	Homeobox genes
ICC	Immunocytochemistry

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Chapter 6

INPs	intermediate neuronal precursors
kb	kilobase pair(s)
LTRs	long terminal repeats
MAP2	Microtubule associated protein 2
Mash-1	Mammalian achaete-scute homolue-1
M-MLV	Moloney murine leukaemia virus
N2 medium	serum-free growth medium
NCAM	Neural cell adhesion molecule
NF160	neurofilament 160
NGF(R)	Nerve growth factor (receptor)
neo	neomycin
NSE	neuron specific enolase
NT3/5	Neurotrophic factor 3/5
O/E	Olf/EBF
Olf-1	Olfactory factor-1
OMP	Olfactory marker protein
OP	olfactory placode
OR	olfactory receptor
ORF	open reading frame
PBS	phosphate buffered saline
PDT	population doubling time
PNS	peripheral nervous system
pRb	Retinoblastoma protein
RA	retinoic acid
RARs	retinoic acid receptor(s)
RARE	retinoic acid response elements
rHLH	repeat helix loop helix
RT-PCR	reverse transcriptase polymerase chain reaction
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
SV40 Tag	Simian virus 40 large T antigen
TC	tissue culture
TGF	Transforming growth factor
TM	transmembrane
ts	temperature sensitive
U	units of enzymes
VNO	vomeronasal organ

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Chapter 1

General Introduction

1.1 The olfactory epithelium

Neural cell progenitors differentiate in response to complex extracellular and environmental cues. The olfactory neuroepithelium provides a convenient experimental system for the study of these interactive signals in that it is the only true neuronal system in vertebrates in which neurogenesis occurs throughout the life of an animal (Cuschieri & Bannister 1975; Graziadei & Monti Graziadei 1978b).

1.1.1 *The origin of the olfactory epithelium*

During embryogenesis the ectoderm invaginates to form the neural tube. During closure of the neural tube two specialised epithelial thickenings develop at the rostral end of the head to form the olfactory placodes. In the mouse embryo, these appear early in the second week of gestation as a thickening of the ectoderm on the ventrolateral side of the head. On the 10th day of gestation the placodes invaginate to form the olfactory pits (Cuschieri & Bannister 1975, Le Douarin *et al.* 1986) which are separated from each other by the nasal septum.

The first sign of cellular differentiation within the olfactory placodes occurs on the 10th day of gestation when axons of the olfactory nerve develop from the base of the olfactory epithelium. Between the 10th and the 13th embryonic days (E10-E13), most of the important features of the mature olfactory epithelium are established and it is possible to detect differentiation and layering of nuclei within the olfactory epithelium (Cuschieri & Bannister 1975).

1.1.2 *The structure of the pseudostratified olfactory epithelium*

The mature olfactory epithelium is a pseudostratified, columnar mucous membrane comprising several laminar zones in which are located the various epithelial cell types (Fig 1.1). The olfactory epithelium is considered pseudostratified rather than stratified because each cell maintains direct contact with the basement membrane by very slender processes (Farbman 1992). The three major cell types seen are: the olfactory receptor neurons (about 75-80% of the epithelial cell population); the supporting cells (about 15-17 % of the cells); and the basal cells (most of the remainder). In addition, there are a few minor cell types (no quantitative data) also present (Farbman *et al.* 1988).

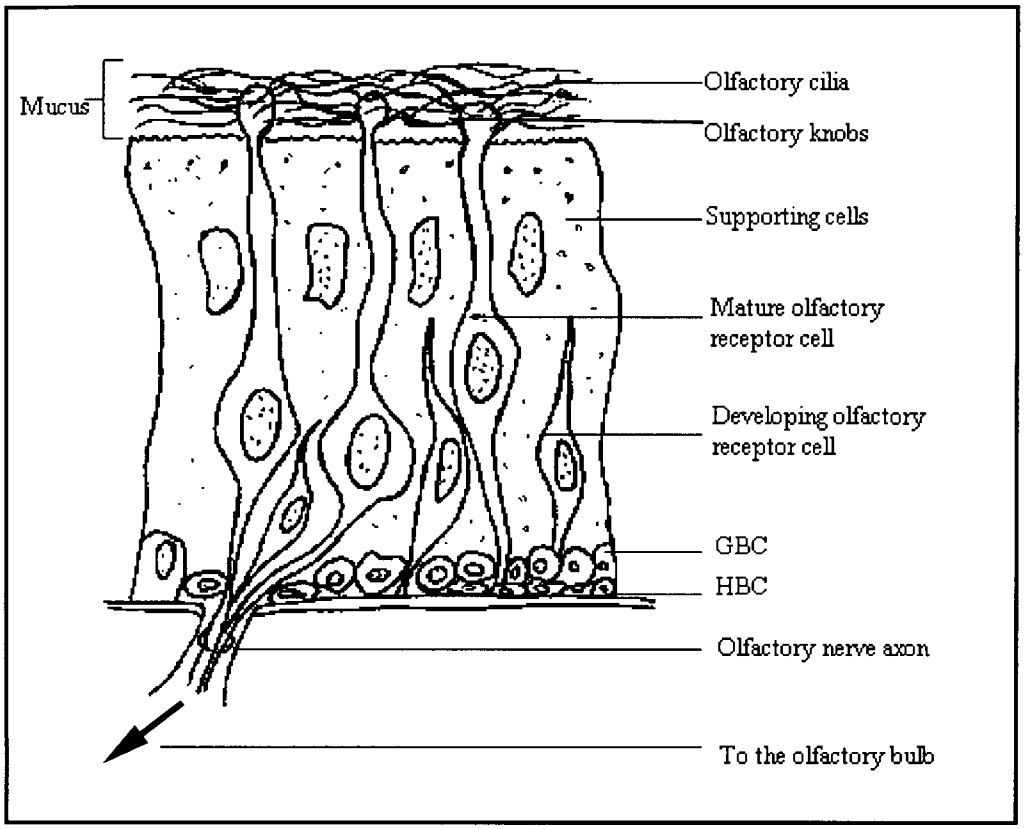


Fig 1.1 The anatomical organisation of the mammalian olfactory epithelium.

The mature olfactory epithelium is a pseudostratified, columnar mucosal membrane comprising of different zones of different cell types: The basal cells: horizontal basal cells (HBC) and the globose basal cells (GBC), the developing olfactory receptor cells, the mature olfactory receptor cells which extends axons to the olfactory bulb and ciliated dendrites to the mucosal surface. The cilia contain odorant receptors which interact with odorants from the external environment. The signal generated by these events are propagated along olfactory receptor neurons to the olfactory bulb. The sustentacular/supporting cells are closest to the mucosal membrane.

1.1.2.1 The sustentacular cells

The sustentacular or supporting cells constitute the most superficial layer from which they extend processes to the basal lamina. Their origin is not clear. Carr *et al.* (1991) believe that they are derived from non-neuronal ectoderm while others are of the opinion that they originate from the horizontal basal cell layer or a subpopulation thereof (Davis & Reed 1996, Suzuki & Takeda 1991b).

The following functions have been attributed to the olfactory supporting cells:

- The fact that they contain the smooth endoplasmic reticulum - associated cytochrome P-450 system suggests that they are responsible for detoxifying and degrading olfactory stimuli (Dahl *et al.* 1982).

- Because of the abundant array of smooth endoplasmic reticulum, which is known to be involved in fluid and ion transport, supporting cells are thought to participate in the regulation of the ionic composition of the mucus layer (Getchell *et al.* 1984).
- Their position and extent suggest a regulatory role for the passage of substances between the epithelial surface and the lamina propria (Rafols & Getchell 1983).
- Because they express ubiquitin and the heat shock protein HSP-70 in response to stress (Carr & Farbman 1991), it has been postulated that they have a local protective function.
- Goodman *et al.* (1993) and Pixley (1992) have suggested that they play a role in the development of the primary olfactory system.

Each sustentacular cell, with its distinguishing oval nucleus, is surrounded by the dendrites and somata of 2-8 sensory olfactory receptor neurons (Graziadei & Monti Graziadei 1979) with their round nuclei.

1.1.2.2 Olfactory receptor neurons

The olfactory receptor neurons line the nasal cavity to form a sensory epithelium of true neurons that extend axons to the CNS and dendrites to interface with the external environment. These neurons, being exposed directly to the external environment, lack the protection enjoyed by neurons elsewhere and for this reason have to be replaced continually in order to maintain olfactory perception throughout the lifespan of the animal. The average lifespan of the olfactory receptor neuron is 30 days (Graziadei & Monti Graziadei 1979; Moulton 1975) although, in the absence of injury or disease-related destruction, they may survive for at least 12 months (Hinds *et al.* 1984). It is a remarkable feature of the olfactory system that continual replacement of receptor neurons has no effect on the overall odor perception.

The nuclei of the olfactory receptor neurons are found in the middle region of the epithelium where they make up 6-8 cell layers. These cells are separated from one another by the cytoplasm of the supporting cells (Graziadei & Monti Graziadei 1979; Farbman 1992). The olfactory receptor neurons in the middle region occupy one of two zones:

The mature olfactory neurons are found in the zone closest to the mucosal surface. They are bipolar neurons with single ciliated dendrites that extend to the mucosal surface. These dendrites are the sites of primary transduction for odorants from the environment.

The immature neurons in the basal half of the middle zone grow dendrites towards the mucosal surface (Caggiano *et al.* 1994). These two types of neurons can be further distinguished ultrastructurally (Cuschieri & Bannister 1975; Graziadei & Monti Graziadei 1979) and immunohistochemically.

Immature neurons express B50/GAP43 (Verhaagen *et al.* 1989), a neuron specific phosphoprotein, which is down-regulated as the olfactory neurons mature and express olfactory marker protein (OMP), a protein associated with their terminal differentiation (Farbman & Margolis 1980; Margolis 1993). Both the mature and the immature neurons express NCAM, a neural cell adhesion molecule. NCAM is used as a marker to distinguish the olfactory neurons from their direct precursor cells, the globose basal cells, which are NCAM-negative (Calof & Chikaraishi 1989).

1.1.2.3 The basal cells

The olfactory basal cells can be divided into two populations on the basis of their position in the olfactory epithelium, the shape of their nuclei and keratin immunoreactivity.

The basal cells situated adjacent to the basal lamina (horizontal basal cells) have nuclei that are flat and elongated (Fig 1.1) and are immunoreactive with antikeratin antibodies (Vollrath *et al.* 1985; Suzuki & Takeda 1991a; b; 1993). On the basis of the autoradiographic studies with [^{3}H] thymidine on the rat olfactory epithelium, Graziadei and Monti Graziadei (1979) have regarded the horizontal basal cells as the self-renewing stem cells of the olfactory cells and the globose basal cells as possible transitional forms between the horizontal basal cells and the olfactory cells. This viewpoint has been supported by some (Guillemot *et al.* 1993; Harding *et al.* 1977; Satoh & Takeuchi 1995; Schwartz Levey *et al.* 1991) while others believe that the horizontal basal cells are not the olfactory neuron precursors (Caggiano *et al.* 1994) but they, or one of their subpopulations, give rise to sustentacular cells (Davis & Reed 1996; Suzuki & Takeda 1991b).

The globose basal cells - also referred to as the intermediate neuronal precursors (INPs) (Calof & Chikaraishi 1989) have nuclei that are round and polyhedral (Graziadei & Monti Graziadei 1979). They are situated above the horizontal basal cells in the olfactory epithelium (Fig 1.1) and are negative for keratin immunoreactivity. They have been shown to be the direct precursors of the olfactory receptor neurons (Caggiano *et al.* 1994; Graziadei & Monti Graziadei 1978a; b; 1979; Calof & Chikaraishi 1989; Schwartz Levey *et al.* 1991; Suzuki & Takeda 1991a; 1993).

By means of *in vitro* quantitative [^{3}H] thymidine uptake experiments performed in combination with immunochemical analysis, Calof and Chikaraishi (1989) demonstrated that the INPs are the immediate precursors of the olfactory receptor neurons. The INPs are morphologically and antigenically distinct from the horizontal basal cells and the olfactory receptor neurons. Pulse-chase [^{3}H] thymidine uptake autoradiography combined with anti-NCAM immunochemical analysis of olfactory epithelium cultures demonstrated that INPs do not express NCAM. INPs divide a few times *in vitro* to give rise to two daughter cells which express NCAM.

To identify the neuronal stem cell in the olfactory epithelium, Calof *et al.* (1998) isolated olfactory epithelium progenitors (mainly INPs) from LacZ expressing mice, cultured them over monolayers of feeders cells and then analysed colonies derived from these co-cultures. One of these colony types consisted of differentiated olfactory receptor neurons ($NCAM^+$) and undifferentiated, proliferating progenitor cells ($NCAM^-$ and labelled with [^{3}H] thymidine). These authors thus proposed that these neuronal colonies arose from a neuronal colony forming cell or unit (CFU). This CFU has the capacity for self-renewal and the ability to give rise to a lineage whose end point is a terminally differentiated cell - and is thus the stem cell in the olfactory neuronal lineage. Calof *et al.* (1998) proposes that the CFU is exclusively neuronal and that the non-neuronal cells have an independent pathway.

The early *in vivo* reports using [^{3}H] thymidine autoradiography suggested that horizontal basal cells divide and give rise to new olfactory cells via globose basal cells (Graziadei & Monti Graziadei 1979). Combining immunohistochemistry of keratin and bromo-deoxyuridine (BrdU) method to label dividing cells, Suzuki & Takeda (1991) have reported that globose basal cells increase their mitotic activity after axotomy, but horizontal basal cells do not. From this the authors inferred that the globose basal cells are the stem cells of the olfactory cell population. Similar observations have been made in bulbectomised mice using immunohistochemistry of keratin and [^{3}H] thymidine autoradiography (Schwartz Levey *et al.* 1991).

To examine the division of globose basal cells and horizontal basal cells in the olfactory epithelium during development, Suzuki & Takeda (1993) used E18 mouse embryos, mice of postnatal days 1-21 and adult mice with double immunostaining of anti-BrdU and anti-keratin antibodies. These *in vivo* studies suggested that during late embryonic and postnatal days, olfactory cells originate from globose basal cells, not from horizontal basal cells. A small number of BrdU-labelled horizontal basal cells was observed in the olfactory epithelium, but the authors excluded the possibility that the horizontal basal cells gave rise to the globose basal cells on the basis of their previous olfactory

receptor molecule. *In situ* hybridisation analysis with RNA probes prepared from these clones showed that the receptor subfamilies were expressed in distinct zones in the olfactory epithelium. The zones exhibited bilateral symmetry in the two nasal cavities and were organised along the dorso-ventral and the medio-lateral axes. These zones were mutually exclusive with only slight overlap at shared boundaries.

Neurons that expressed a given receptor gene were confined to the same zone but were randomly distributed throughout that zone. Receptors which recognised related odorants were also expressed in the same zone (Ressler *et al.* 1993; 1994b).

1.2.5 *Axons of the olfactory receptor neurons converge on the olfactory bulb in an organised fashion*

To determine whether olfactory receptor neurons connect to defined regions in the olfactory bulb, Ressler *et al.* (1994a) used *in situ* hybridisation with olfactory receptor probes to show that neurons which expressed the same receptor projected their axons onto a small number of glomeruli in the olfactory bulb. The glomeruli were identified by counterstaining the sections with a fluorescent nuclear dye to identify their characteristic features. The axonal projections from neurons expressing a specific receptor converge upon two of 1800 glomeruli within the olfactory bulb. Neurons expressing a given receptor project to one medial and one lateral glomerulus, creating a mirror image map within each olfactory bulb (Ressler *et al.* 1994a; Vassar *et al.* 1994). The expression of an olfactory receptor gene is required for the axon to find its way to the glomeruli. Neurons first choose their receptors and then find their target in the olfactory bulb (Mombaerts 1996; Mombaerts *et al.* 1996).

Ressler *et al.* (1994a) suggest that since olfactory receptors are highly diverse, especially in the ligand binding domain, different glomeruli are likely to recognise different determinants on an odorant. Olfactory receptor neurons in different expression zones in the olfactory epithelium project their axons to the olfactory bulb in such a way that neurons in the dorsal region of the nasal cavity project to the dorsal bulb regions and ventrally located neurons project to the more ventral bulb regions (reviewed in Sullivan *et al.* 1995). Thus it appears that the zonal organisation of sensory information in the olfactory epithelium is maintained in the transmission of this information to the olfactory bulb.

The I7 receptor is expressed on 0.2% of cells present in the ventral domain of the olfactory epithelium. This domain occupies approximately 20% of the nasal epithelium. One may calculate, therefore, that approximately 1 cell in 2500 taken from the nasal turbinates of individual F1 animals should be I7-positive. A sample of 200 such cells would have a mean likelihood of 8% of containing a single cell and a probability of less than 1% of containing more that more than one cell. These theoretical values agreed well with their findings that only 9 of 90 pools of 200 cells contained, by RT-PCR, an I7 transcript - presumably from a single cell. Analysis of the cDNA from this transcript showed that they were either of the paternal **or** maternal origin; not both. Similar results were obtained with a different olfactory receptor, I54 and the screening of M13 subclone of PCR products of single cells showed that plaques hybridised exclusively to either the *Mus Musculus* or the *Mus Spretus* I7 probe. They concluded that in the F1 hybrid one of the parental alleles was transcriptionally silenced or excluded.

When replication of olfactory receptor genes was studied in interphase nuclei, a high percentage (31-39%) of alleles showed asynchronous replication. This is a consistent feature of alleles that are differentially regulated in such a way that one is transcriptionally inactive.

The authors suggest the following plausible model for olfactory receptor gene expression in a given neuron: Early in embryogenesis one parental allele is stochastically inactivated. The remaining allele contain clusters of genes, a subset of which may bind to gene specific transcription factors that give them the potential to be expressed in that zone, while the expression of other subsets is permitted only in other zones. A cis-acting regulatory element (possibly a strong enhancer, or a recombination site) then determines - once again in a stochastic manner - which of the permitted genes in the array may be expressed in the cell.

1.2.4 *Zonal organisation of the olfactory epithelium*

In situ hybridisation studies designed to discern the pattern of receptor expression across the olfactory epithelium have shown that the nasal cavity is divided into at least four distinct zones in which different sets of olfactory receptors are expressed (Ressler *et al.* 1993; Vassar *et al.* 1993).

Ressler *et al.* (1993) used PCR-generated probes of the coding regions of mouse olfactory receptor genes to isolate three genomic clones (K4, K7 and K18) that hybridised to multiple bands in Southern blot analysis but did not cross-hybridise with each other. Each clone represented a subfamily of 10-15 highly homologous genes each, in turn, encoding a highly related odorant

Members of this olfactory receptor family show greatest sequence diversity in the TM3, 4, and 5 - an observation that is consistent with these being potential ligand binding domains capable of binding to a large array of odorants. Motifs conserved among members of the family of olfactory proteins included GN in TM1; the central W of the TM4; the Y near the C-terminal end of TM5; and the NP in TM7. Within the family of olfactory receptors, subfamilies have been identified. Individual genes in the olfactory family share 40-80% identity, while receptors which are 70-80% identical to each other belong to the same subfamily (Buck & Axel 1991; Ressler *et al.* 1994b).

It was originally believed, on the basis of northern blot analysis, that expression of the olfactory gene family was confined to the olfactory epithelium (Buck & Axel 1991). However, subsequent studies have identified transcripts of similar genes in sperm (Parmentier *et al.* 1992; Vanderhaeghen *et al.* 1993) and in cardiac tissue (Drutel *et al.* 1995).

Southern blots of genomic DNA and screens of genomic DNA libraries indicated that the gene family consisted of 100 or more members (Buck & Axel 1991); later studies have increased the estimate to between 500-1000 in rodents (Chess *et al.* 1994; Ressler *et al.* 1994b).

1.2.3 *Allelic inactivation: one neuron - one receptor*

Olfactory odorant receptors are encoded by linked multigene clusters in the genome (Lancet *et al.* 1993; Ben-Arie *et al.* 1994; Reed 1992). Chess *et al.* (1994) that include, in mammals, as many as 1000 different genes. These are uniquely expressed - both in terms of their topological location in the nasal epithelium and inasmuch as each cell expresses only a single receptor. Statistical arguments have supported the hypothesis that each olfactory receptor neuron is commited to only one olfactory receptor subtype (Ngai *et al.* 1993b) and for that subtype, only one allele (Chess *et al.* 1994). There are thus clearly mechanisms that exist to control transcription of this large family.

The experiments of Chess *et al.* (1994) have been very informative in this regard. They used for their studies, a polymorphic receptor, I7, that is encoded by a single gene on chromosome 7 of the mouse. The alleles in *Mus Musculus* and *Mus Spretus* differ in 3 sites in the 5' untranslated region of the gene and 23-mer oligonucleotides spanning one such site could be used to distinguish clearly the two I7 alleles and hence paternal and maternal I7 RNA transcribed in cells of the F1 hybrid.

Teeter 1990). Raming *et al.* (1993) also showed in a preliminary study that Sf9 insect cells transfected with a putative olfactory receptor responded to odorants with a dose dependent increase in IP_3 formation.

Evidence for odor responses mediated by opening of IP_3-gated channels are found in invertebrates (spiny lobster *Panulirus argus* olfactory receptor neurons). Fadool & Ache (1992) showed that an inward current induced by a mixture of odorants in cultured lobster olfactory receptor neurons is enhanced fourfold by addition of an antibody raised against the COOH terminus of the mammalian IP_3 receptor.

Studies indicate that there may be more that one olfactory transduction pathway and this could lead to increased odorant discriminability and detectability.

1.2.2 *Cloning of the olfactory receptor genes*

Buck and Axel (1991) cloned the first olfactory receptors from rat DNA. They used the known features of odorant signal transduction to predict the likely molecular features of the proposed odorant receptor molecules and they based their strategy on these predictions. It was known, for example, that cAMP served as a second messenger for odorant detection and that GTP was necessary for mediating the odorant-induced increase in cAMP levels. They therefore assumed that the odorant receptor molecules belonged to the superfamily of receptors coupled to the GTP-binding proteins. They used PCR with degenerate primers targeted to conserved regions of the GPCR superfamily in the transmembrane TM2 and TM7 regions to amplify candidate genes from olfactory cDNA.

To determine whether the PCR products were derived from a mixture of DNA sequences and hence a representative of a multigene family, they digested these products with restriction enzymes that cut with a high frequency. Bands related to a single gene gave restriction products whose sum matched the weight before digestion, but some bands yielded products summing to much higher weights, indicating the presence of a multiple gene family. When the genes were cloned and sequenced, they were found to form a multigene family with seven TM domains. These clones formed a family within the superfamily of G-protein linked receptors since their sequences were much more homologous to each other than they were to other previously known members of this superfamily.

The effector protein for G_s, G_i and G_{olf} is AC; the effector protein for G_o is unknown (Jones & Reed 1989; Gilman 1984; 1987). The enzyme AC catalyses the synthesis of cyclic adenosine 3', 5'-monophosphate (cAMP) from adenosine triphosphate (ATP) and is stimulated by certain odorants in a dose-dependent manner (Sklar *et al.* 1986). cAMP acts as an intracellular messenger in the transduction by opening a conductance channel. This causes depolarisation of the cell membrane and the generation of a nerve impulse (Gold & Nakamura 1987). Activation from an inhibitory G-protein inhibits AC activity with reduced production of cAMP and nullification of the response by hyperpolarisation of the cell membrane (Michel & Ache 1992).

Certain odorants that do not stimulate AC are believed to use another transduction mechanism that involves the phosphoinositide system. In this system the enzyme phospholipase C is activated. This promotes the breakdown of inositol bisphosphate (PIP_2) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). These act as second messengers that trigger a cascade that initiates or inhibits a nerve impulse (Huque & Bruch 1986; Restrepo *et al.* 1990).

Studies of odorant responsivity suggest mediation by cAMP or IP_3 on the basis of pharmacology, genetic manipulation, ionic dependence or biochemical measurements. The evidence for IP_3 as a second messenger in the signalling of vertebrate olfactory receptor neurons is still controversial and much less analysed than the cAMP-mediated pathway. The involvement of IP_3 in invertebrates are more conclusive (reviewed by Schild & Restrepo 1998).

The cAMP-gated channel was first detected in excised patches from olfactory cilia of toad (*Bufo marinus*) olfactory receptor neurons by Nakamura & Gold (1987). These authors reported activation of non-specific cation channels by cAMP in excised patches from olfactory cilia membranes. The evidence in support of the mediatory role of cAMP-gated channels in vertebrate olfactory transduction is conclusive. A cAMP-gated channel-deficient transgenic mouse is anosmic to all odorants tested as assessed by electroolfactogram (EOG) measurements (Brunet *et al.* 1996). This led the authors to conclude that cAMP is the only second messenger mediating olfactory transduction.

Huque & Bruch (1986) first determined that certain amino acids known to be potent odorants in catfish elicit increases in breakdown of PIP_2, IP_3 and DAG in a GTP-dependent manner. Biochemical experiments with isolated olfactory cilia from catfish indicate that amino acid odorants stimulate formation of IP_3 (Bruch & Huque 1986; Restrepo *et al.* 1993), although formation of cAMP was also stimulated at higher concentrations and after long exposure to the odorant (Bruch &

receptor (Martens 1992). The binding of the ligand leads to a conformational change in the binding pocket of the receptor. These changes result in coupling and activation of the G-protein.

The involvement of GPCR in the olfactory transductory pathway is based upon the expression of G-proteins in cilia at the time that these structures first appear in the olfactory receptor neurons - E16 in rats (Menco & Farbman 1985) or E13 in mice (Noda & Harada 1981) - and coincident with the first recordable action potentials (Mania-Farnell & Farbman 1990). Furthermore, olfactory adenylate cyclase (AC) that is found abundantly in the cilia can only be activated in the presence of guanosine triphosphate (GTP) (Pace *et al.* 1985; Pace & Lancet 1986).

The olfactory epithelium contains 3 types of G-proteins; stimulatory G-proteins (G_s), inhibitory G-proteins (G_i) and G-proteins 'other' (G_o). They are all hetrotrimers composed of a guanyl nucleotide binding α-subunit, a β-subunit and a γ-subunit. The α-subunits are situated near the binding sites for receptor and effector proteins (Farbman 1992).

Two relatively specific olfactory G-protein subunits have been identified; the $G_{olf\alpha}$ and Gγ8. It was originally thought that $G_{olf\alpha}$ was exclusively expressed by mature sensory neurons in olfactory tissue (Jones & Reed 1989) but subsequently its expression has been detected in basal ganglia where it is thought to couple D1 dopamine receptors to AC (Drinnan *et al.* 1991), and $G_{olf\alpha}$ mRNA has been found in testis, retina, brain and liver (Zigman *et al.* 1993), albeit at a lower levels than in the olfactory epithelium.

The GTP-binding protein, Gγ8, is specifically and prominently expressed in immature neurons of the olfactory epithelium and the vomeronasal epithelium (Ryba & Tirindelli 1995). Its expression decreases in mature neurons, suggesting a role in olfactory neurogenesis. The expression of Gγ8 in mature neurons is 10 fold lower than that of $G_{olf\alpha}$. These two subunits are, therefore, unlikely to form a complex involved in signal transduction in the mature olfactory receptor neurons.

As a general rule, G-protein coupled receptors transduce external signals by undergoing a conformational change in response to activation by their ligands. This stimulates an effector protein (Neer & Clapham 1988) that is exposed at the cytoplasmic surface of the cell membrane. The effector protein is often an enzyme, e.g., AC, phospholipase C or phospholipase A2 (Burch *et al.* 1986; Krupinski *et al.* 1989).

studies have shown that OMP - a marker of olfactory neuron maturity - expression can be induced in olfactory neurons without specific bulb contact, but by contact with such tissue as non-bulb brain tissue (Magrassi & Graziadei 1996), disaggregated nasal epithelial cells or monolayers of central nervous system (CNS) astrocytes (Pixley 1992).

Cells dissociated from the developing and adult olfactory organ when transplanted into the rat fetal brain can either completely change their fate and differentiate according to their final position or generate an olfactory epithelium, if they reaggregate into large clusters (Magrassi & Graziadei 1996). This evidence is supported by Goldstein *et al.* (1998) who showed that grafted precursors harvested from bulbectomised donors produced both neuronal and non-neuronal cells suggesting that epithelial precursors were not irreversibly committed to making neurons. They thus suggested that olfactory progenitors were subject to a form of feedback control *in vivo* that regulated the types of cells that they produced within a broader-than -neuronal repertoire.

1.2 Olfactory receptors

The olfactory system has the remarkable capacity to discriminate among a wide range of odorant molecules. This process begins in the olfactory receptor neurons that convert information contained in the odorant molecules into information contained in membrane signals and neural space.

The initial events in the odor discrimination occur with the binding of odorants to specific olfactory receptors on the cilia of the dendrites of the olfactory neurons. The binding of odorants to these receptors initiates an electrical signal that travels along the axons to the glomeruli in the olfactory bulb that serves as the first relay station for the processing of olfactory information in the brain. The bulb then connects the nose with the olfactory cortex, which then projects to higher sensory centres in the cerebral cortex and the area of the brain that controls behaviour (Farbman 1992).

1.2.1 *Olfactory receptors belong to the family of G-protein coupled receptor*

The olfactory receptors belong to the superfamily of guanine nucleotide binding protein (G-protein) coupled receptors (GPCR) (reviewed in Martens 1992; Probst *et al.* 1992). GPCR are structurally similar with seven putative membrane spanning domains, each consisting of approximately 20-25 hydrophobic amino acid residues (Lefkowitz & Caron 1988). The ligand binding sites for many GPCR have been shown to lie within the TM domains and induce ion pairing, hydrogen bonding and hydrophobic interactions between portions of the ligand and specific amino acid residues of the

Bowman's glands provides most of the mucus covering the olfactory epithelial surface (Farbman 1992).

1.1.3 *Connection between the olfactory neurons and the olfactory bulb*

New sensory neurons are continually produced by mitotic division in the globose basal cell layer (Calof & Chikaraishi 1989, Graziadei & Monti Graziadei 1978a; Schwartz Levey *et al.* 1991; Suzuki & Takeda 1991a). Following mitosis a daughter cell moves apically in the epithelium while developing a single basally directed axon and an apical dendrite. The dendrite reaches the epithelial surface and sprouts cilia from its knob-like terminus. The axon extends through the basement membrane and projects to the olfactory bulb. Final maturation occurs as the axon forms a synapse in the bulb (Calof & Chikaraishi 1989; Carr & Farbman 1993; Schwartz Levey *et al.* 1991).

Early olfactory axons grow from the epithelium to the telencephalon along a highly specific pathway. Since the initial olfactory axons do not appear to contact olfactory Schwann cells (Gong *et al.* 1994), the guidance cues for early axon outgrowth are believed to come from extracellular matrix molecules (e.g. laminin) and cell surface molecules (e.g. NCAM and L1) (Gong & Shipley 1996).

Laminin is an extracellular matrix molecule that is produced by astrocytes and is expressed during embryogenesis and in the adult. L1 is a cell-surface molecule that is expressed in immature, but not mature, olfactory neurons. L1 immunoreactivity is strongest in the olfactory nerve, suggesting an important role in axonal extension and, possibly, in the differentiation of the olfactory neurons (Gong & Shipley 1996). The neural cell adhesion molecule (NCAM), which is a cell surface molecule, has been shown to be present on the surface of the olfactory neurons and their axons; it is important for axon-axon interaction within the olfactory nerve and for olfactory axon outgrowth in the adult animal. Rourke (1996) have proposed that the 180 kDa polysialylated form of NCAM plays an important role in the migration of neuroblasts into the olfactory bulb. The polysialylation would serve to weaken cell-cell interactions and allow the neuroblast to translocate through the "local" micro-environment (Ono *et al.* 1994; Rourke 1996).

The olfactory neurons reach maturation once they make contact with the olfactory bulb. It has been proposed that complete differentiation of olfactory neurons require the presence of an intact olfactory bulb - a theory that is supported by data from explant cultures (Chuah *et al.* 1985), bulbectomy and nerve section studies (Harding *et al.* 1977; Schwartz Levey *et al.* 1991). Other

Stem cells are those from which other types of cells arise. The most primitive of stem cell of all is the fertilised egg - from this single cell arise the many cell types that constitute a mature multicellular animal. However, the term stem cells has been used to describe cells with far less developmental potential. In a given tissue one may speak of stem cells that have the ability to give rise to all of the differentiated cell types associated with that specific tissue.

A stem cell may then be defined as a cell:

- that is capable of extended self-renewal throughout the life of an animal,
- that is not in the final stage of differentiation or terminally differentiated,
- that has the ability to generate multilineage cell types, including neurons, glia and other cell types present within the CNS (e.g. interneurons, projecting neurons, type I astrocytes), and
- that has progeny that can either continue as stem cells or terminally differentiate. A stem cell divides asymmetrically to produce one daughter cell like itself and one daughter cell that divides to produce differentiated cells (Gage *et al.* 1995).

The progeny of stem cells are termed progenitor cells. These are also capable of self-replication but only for a limited number of cell divisions. There is a hierarchy of progenitor cells with respect to lineage potential but progenitors generated later in ontogeny show lineage restriction and, ultimately, commitment to a single lineage (Kilpatrick *et al.* 1995).

In the CNS the two fundamental cell types are neurons and glial cells. On this basis, neural progenitor cells fall into three classes:

1. cells that can generate both neurons and glial cells (neuron/glial progenitor cells)
2. progenitor cells that generate only neurons (neuroblasts)
3. progenitor cells that generate only glial (glioblasts)

Neuroblasts and glioblast may be pluripotential, generating more than one type of neuronal cell or glial cell, respectively. Alternatively, they may be unipotential, generating only one type of progeny. Such cells are sometimes referred to as 'precursor cells' denoting their pre-ordained fate (Temple & Qain 1996).

1.1.2.4 Bowman's glands

Bowman's glands lie in the lamina propria below the epithelial basement membrane and give rise to ducts that penetrate through the epithelium and open onto the mucosal surface. Secretion from the

axotomy experiments (Suzuki & Takeda 1991) and the fact that the BrdU-positive horizontal basal cells did not correspond to the BrdU-positive globose basal cells.

The data presented above concentrates on the destruction and replenishment of neurons in the olfactory epithelium and does not necessary apply to conditions in which both the neurons and non-neuronal cells of the epithelium have been depleted.

Huard *et al.* (1998) used retroviral vectors to study the lineage relationship of cells in the adult olfactory epithelium after methyl bromide (MeBr) lesions, which destroys neurons, sustentacular cells, some Bowman`s duct/gland cells but proliferating cells (globose basal cells, horizontal basal cells and Bowman`s gland cells) are unaffected and thus become the target for infection by and integration of the vector. The authors proposed the existence of multiple types of globose basal cells along the olfactory lineage, after MeBr destruction. They suggest that a sub-population of globose basal cells is the self-renewing cells (and thus the stem cell) in this system and this stem cell gives rise to a multipotential globose basal cell which can generate neurons, sustentacular cells and horizontal basal cells. However, in normal or bulbectomized olfactory epithelium, this multipotential globose basal cell is neuripotent giving rise only to olfactory receptor neurons and its ability to generate horizontal basal cells and sustentacular cells is "blocked". The pathway from the ducts to the sustentacular cells is also blocked in the normal olfactory epithelium. But in MeBr treated olfactory epithelium, the ducts give rise to Bowman`s gland cells and sustentacular cells.

Thus, the adult olfactory epithelium harbours a multipotential cells whose capacity to give rise to neurons and / or non-neuronal cells is activated by which cells need to be replenished.

Further support that non-neuronal cells are also derived from globose basal cells is the finding that some cells in the basal zone of the olfactory epithelium express the globose basal cell marker, GBC-1, along with sustentacular antigens or horizontal basal cell antigens at early stages in the recovery of the epithelium after MeBr lesion (Goldstein & Schwob 1996).

1.1.2.3.1 Neural stem cells and progenitor cells

The subject of neural stem cells and progenitor cells has been well reviewed by a number of researchers in the field including Altman (1996); Gage *et al.* (1995); Kilpatrick *et al.* (1995); Stemple & Mahanthappa (1997); Temple & Qian (1996).

Mombaerts *et al.* (1996) have developed a genetic strategy to visualise axons from olfactory receptor neurons expressing a given odorant receptor as they project to the olfactory bulb. Their experimental strategy was based on the targeted integration of the axonal marker tau-lacZ downstream of the coding sequence of an olfactory receptor gene in such a way that this reporter gene is co-expressed with the receptor gene from the mutated alleles. The translation of the reporter is controlled by an internal ribosome entry site (IRES) of viral origin. Neurons expressing the mutated receptor gene also expressed tau-lacZ, which makes their axons turn blue when exposed to X-gal and thus allow direct visualisation of the pattern of projection in the brain.

They have modified an olfactory receptor gene, P2, by targeted mutagenesis in the germline of mice. The P2 locus now encodes a bicistronic mRNA that allows the translation of the P2 receptor along with tau-lacZ (P2-IRES-tau-lacZ).

The results of these genetically manipulated mice suggest that, from the earliest stage in embryonic development, the blue axons appear to project to only two topographically fixed glomeruli in the mouse olfactory bulb. In embryonic P2-IRES-tau-lacZ mice they observed blue neurons expressing receptors that have not elaborated axons, suggesting that neurons first express a given receptor and only later project to specific target in the olfactory bulb.

To study the effect of a receptor swap on axonal projections, the authors replaced the P2 receptor coding sequences with the coding sequence of a second receptor, M12 (M12 → P2). The M12 receptor was under the control of the P2 regulatory sequences. Neurons that chose to express this modified P2 allele instead expressed M12 along with tau-lacZ (M12 → P2- IRES-tau-lacZ), allowing the direct visualisation of the consequence of a receptor swap on the pattern of axonal projection. The authors observed the convergence of axons to topographically fixed glomeruli distant from the wild type M12 glomeruli but quite close to the wild type P2 glomeruli, suggesting that the olfactory receptor plays an instructive role in the guidance process but cannot be the sole determinant in axon targeting.

The linkage observed between the choice of an odorant receptor and the site of axonal convergence in the olfactory bulb led the authors to propose the following model:

"Perhaps the most parsimonious model would argue that the odorant receptors is expressed on dendrites and recognises odors in the environment, and is also expressed on axons termini, where it would recognise a set of guidance cues, distinct from odorous ligands, expressed by bulbar cells. In

this manner, an olfactory neuron would be afforded a distinct identity that dictates the nature of the odorants to which it responds, as well as the glomerular target to which its axons project".

In the mouse there are about 204 million olfactory receptor neurons in each nasal cavity (MacKay-Sim & Kittel 1991) whereas there are only 1-2 thousand glomeruli in each olfactory bulb (Royet *et al.* 1988). Given that there may be 1000 different odorant receptor genes in the mouse, each glomerulus is expected to receive input from a large number of olfactory sensory neurons. This would imply that olfactory perception is broadly organised in the nose and further organisation is achieved in the olfactory bulb, the first relay station for processing olfactory information.

1.3 The VNO and the pheromone receptors

In mammals, olfactory stimuli are detected by sensory neurons at two distinct sites: the olfactory epithelium of the nasal cavity and the neuroepithelium of the vomeronasal organ (VNO). While the olfactory epithelium can detect volatile chemicals released from numerous sources, the VNO is specialised in that in most terrestrial vertebrates, it mediates only the detection of pheromones related to social and reproductive behaviour. Pheromones are chemical substances that are secreted or excreted by an individual and have an endocrinological effect on other members of the same species. A number of these effects are described in the review by Halpern 1987. In brief they include:

- Suppression of estrus in group-housed females (Lee-Boot effect).
- Acceleration of estrus and estrus synchrony produced by male odors (Whitten effect).
- Acceleration of puberty in female mice produced by male odors (Vandenbergh effect).
- Pregnancy block caused by odor of a 'strange' male (Bruce effect).

Keverne & de la Riva (1982) suggested that pheromones induce a change in hypothalamic dopamine turnover which in turn affects the release of GnRH and prolactin inhibiting factor. In general, male pheromones are thought to reduce prolactin levels and increase lutenizing hormone, while female pheromones are thought to increase prolactin secretion (Halpern 1987).

The VNO (Fig 1.2) in rodents is a cartilage-encased tubular structure, located at the base of the nasal septum, that opens into the nasal cavity via a single duct. The vomeronasal neuroepithelium consist of three types of cells; supporting cells, sensory cells and undifferentiated basal cells. [^{3}H] Thymidine incorporation suggests that the basal cells give rise to sensory cells in the mice (Barber & Raisman 1978; Wilson & Raisman 1980). The vomeronasal neuroepithelium contain sensory

neurons that project axons to the accessory olfactory bulb (AOB) of the brain (Barber 1981) along a pathway that is different from that which conveys sensory information from the olfactory epithelium to the main olfactory bulb (MOB) (Price 1987). Olfactory epithelium-derived signals ultimately reach many regions of the brain, including the frontal cortex, so mediating the conscious perception of odors. In contrast, VNO-derived signals are targeted to hypothalamic structures implicated in reproductive physiology (Kevetter & Winans 1981; Krettek & Price 1978).

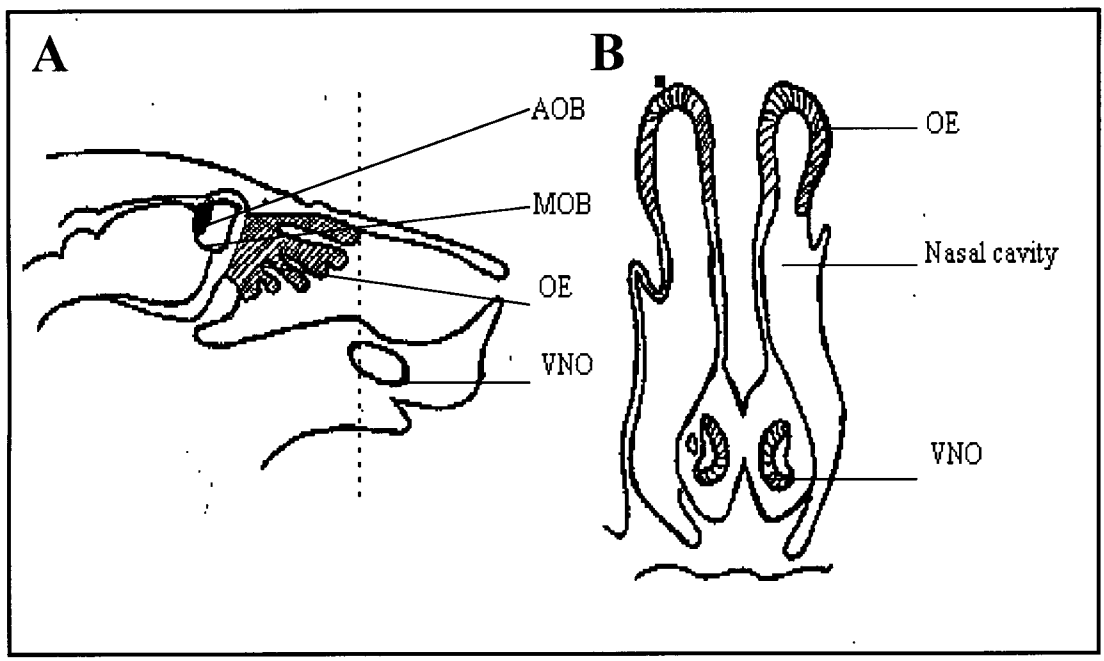


Fig 1.2 *Anatomy of the mouse olfactory system.*

A] A schematic drawing of a parasagittal section showing the VNO (vomeronasal organ) and the turbinates of the OE (olfactory epithelium), which resides within the posterior recess of the nasal cavity, whereas the VNO resides more anteriorly in a blind-ended pouch within the septum of the nose. The VNO projects to the AOB (accessory olfactory bulb), whereas the OE projects to the MOB (main olfactory bulb). B] In a section taken at the position indicated in (A) with a dashed line, shows the anatomically distinct VNO and the OE (Berghard *et al.* 1996; Dulac & Axel 1995; Liman 1996).

The neuroepithelium of the VNO is derived embryologically from an invagination of the developing olfactory epithelium which it resembles morphologically (Cuschieri & Bannister 1975). Both VNO and olfactory epithelium neurons are regenerated continually throughout life from a population of basal cells (Barber & Raisman 1978, Graziadei and Monti Graziadei 1979); they are both bipolar neurons that extend fine processes (cilia in the case of olfactory receptor neurons and microvilli in the case of VNO receptor neurons) into the external environment and both project axons to the olfactory bulb of the brain (Price 1987, Graziadei & Monti Graziadei 1979; Miragall *et al.* 1979).

The cilia in the olfactory receptor neurons and the microvilli in the vomeronasal receptor neurons are the sites of sensory transduction and northern blots and *in situ* hybridisation analyses have

shown that different molecules are involved. $G_{olf\alpha}$, AC type III and an olfactory cyclic nucleotide-gated (CNG) channel subunit 1 (oCNC1) are expressed in olfactory neurons but not in those of the VNO whereas oCNC2 is expressed in the VNO. This suggests that the signal transduction pathways for the two systems are different, but it is possible that sensory transduction in the VNO also involves CNG ion channels (Berghard *et al.* 1996).

Dulac & Axel (1995) were the first to clone the putative pheromone receptors in rats. By using differential screening of cDNA libraries constructed from single sensory neurons from the VNO, they were able to isolate a family of 30 putative receptor genes (V1Rs pheromone receptor family). They used reverse transcriptase to generate double stranded cDNA probes and cDNA libraries from individual VNO neurons. By screening the cDNA libraries with clones from the VNO and the olfactory epithelium they isolated receptors which also belonged to the GPCR superfamily and were exclusively expressed in the VNO. This novel family of receptors is not closely related to olfactory receptors in the main olfactory epithelium. The sequences of the olfactory receptors and the VNO receptors share homology, indicating that these two pathways developed independently.

Within a family of pheromone receptors, members share 40-90% sequence similarity with significant differences especially in the TM regions - i.e. the presumed sites of ligand binding. Data from Southern blots and genomic libraries indicate that the multigene family of VNO receptors consist of between 30-40 genes but they further estimate that the repertoire of receptors may consist of approximately 100 genes.

In situ hybridisation has shown that neurons in the VNO express only a single receptor gene and that the neurons expressing a given receptor are randomly distributed along the anterior-posterior axis. Cross-sectional analysis suggest that this receptor family may be restricted to the apical part of the VNO.

The VNO is subdivided into two zones which express different receptors and signalling transduction mechanisms that connect to two different regions in the AOB (Dulac & Axel 1995; Halpern *et al.* 1995; Ryba & Tirindelli 1997). The pheromone receptors belonging to the family isolated by Dulac & Axel (1995) are now referred to as V1Rs. They are expressed in the apical cell layer, which also expresses the G-protein $G_{i\alpha 2}$. The V2Rs, isolated by a second group (Ryba & Tirindelli 1997) are expressed in the basal half of the receptor cell layer that also expresses the G-protein $G_{\alpha o}$.

Ryba & Tirindelli (1997) isolated a second multigene family of receptors by probing a VNO cDNA library with mixed probes of olfactory receptors and found what turned out to be a V2R clone, despite the fact that V2Rs have no sequence homology to olfactory receptors. The V2Rs were similar but not identical to the V1Rs. Unlike the V1Rs and the olfactory receptors, the V2Rs were found to have introns within the coding regions of the genes.

Thus, the olfactory receptors, the V1Rs and the V2Rs are completely unrelated to one another and are derived from different branches of the G-protein-coupled receptor superfamily.

1.4 Unique features of the olfactory system

The olfactory system is unique in several interesting respects:

- Unlike other elements of the CNS system it derives embryologically from the olfactory placode rather than the neural tube or the neural crest (Cuschieri & Bannister 1975).
- The olfactory neurons, unlike other sensory neurons, are superficially located and hence conveniently accessible for experimental studies.
- It is directly exposed to the external environment with a need for all of the biological protective mechanisms that exposure of such a delicate system implies.
- During the normal course of adult life the population of olfactory receptor neurons undergoes attrition by neuronal death with replacement from a pool of constantly proliferating stem cells. The newly generated neurons then develop axons which re-innervate the olfactory bulb and re-establish olfaction (Graziadei & Monti Graziadei 1979, Harding & Wright 1979).
- The capability for functional regeneration of primary olfactory synapses in neonates and adults is remarkable. In other parts of the CNS synapses can be regenerated early in development but not in adulthood.
- Unlike other sensory neurons, the olfactory system projects olfactory information via the olfactory bulb to a cortical region of the brain without first passing through the thalamus. (Farbman 1992; Graziadei & Monti Graziadei 1979).
- In the olfactory epithelium, a 'steady state' in total cell number is achieved by a balance between cell proliferation and cell death. Olfactory neurons are constantly being born from their direct precursors, the globose basal cells. Cells that do not make contact with their targets or are damaged undergo cell death by apoptosis. Neuronal cells at different stages of development have been shown to undergo apoptosis (Carr & Farbman 1993).
- Many cells of the proliferating neuroepithlium of the CNS contain abundant vimentin. When these cells withdraw from the proliferating cell cycle, vimentin expression ceases and

neurofilament proteins accumulate. The olfactory neurons deviate from this general pattern in that, in the adult animal, they continue to express high levels of vimentin while neurofilament proteins are found in only a very small subpopulation of these neurons (Schwob *et al.* 1986).

- The olfactory axons are unmyelinated and are organised in a way that is typical of axons in an early stage of embryonic development in the peripheral nervous system (PNS). The fact that the ensheathing cell-axon relationship remains immature is consistent with the idea that the olfactory cells are not destined for long life and that they contain the signals required for regeneration of neurons (Farbman 1992).
- As they grow and extend from the olfactory epithelium, olfactory axons are directed by molecular signals present on cell surfaces and in the extracellular matrix. In other parts of the brain, axonal migration takes place with Schwann cell guidance (Gong & Shipley 1996).

1.5 *In vitro* studies of the olfactory epithelium

Current knowledge of the biology of the olfactory epithelium has benefited greatly from *in vitro* approaches to the study of this unique structure. For the most part these have involved organotypic cultures of explants, cultures of dissociated cells and the study of cell lines derived from olfactory epithelium by spontaneous or induced transformation.

Although organotypic cultures have a limited lifespan *in vitro*, they do survive for sufficient time for the differentiation of constituent cell types to be examined with such techniques as pulse labelling, immunostaining, histochemistry and conventional histology. They also offer the advantage that cell:cell and cell:matrix interactions that may be important are, to a large extent, preserved.

Studies of olfactory neurogenesis have used explant cultures from the olfactory epithelium of E14-E15 embryos (Calof & Chikaraishi 1989). These cultures were generated from nasal turbinates and the sensory epithelium was purified from underlying stroma by enzymatic digestion and mechanical trituration. When the olfactory epithelium explants were placed in culture in Dulbecco's modified Eagle's medium, two cell types were identified by morphology and immunocytochemistry: a flat epithelial-like keratin$^+$ cell and a keratin$^-$/NCAM$^+$ neuronal cell. To induce these cells to migrate in culture, the olfactory epithelium explants were grown in low calcium, serum-free media. A concentration of calcium 20-fold lower than that found in normal serum caused adherent junctions between epithelial cells to be disrupted. Under these conditions the transient appearance of a migratory, 'round', third cell type was identified. By means of quantitative [^{3}H] thymidine uptake

experiments performed in combination with immunocytochemical analysis, these authors demonstrated that this third cell type was morphologically and antigenically distinct from either the horizontal basal cells or the olfactory receptor neurons and that it represented an intermediate precursor of the olfactory receptor neurons. This they termed the immediate neuronal precursors (INPs). Pulse-chase [^{3}H] thymidine uptake autoradiography, combined with anti-NCAM immunocytochemical analysis of the olfactory epithelium explant cultures, demonstrated that the proliferating INPs do not express NCAM but that their progeny are NCAM$^+$ cells. These results showed that the INPs divided to give rise to two NCAM$^+$ daughter neurons. The fact that the INPs only gave rise to neurons in culture suggested that these cells were already specialised for neuronal differentiation prior to their final mitosis. This culture system did not support complete maturation of the olfactory neurons but supported neuronal differentiation thus implying that other cells present in the culture might be providing signals required for neuronal differentiation. Maturation of these neurons into fully specialised olfactory receptor neurons presumably required other factors such as interaction with the target tissue. Neurogenesis *in vitro* ceased, indicating that existing precursors divided and differentiated but that no new precursors were produced to replace them.

Dissociated cultures from the olfactory epithelium have also been developed in an attempt to isolate olfactory receptor neurons to study neurogenesis in the olfactory system (Pixley & Pun 1990). Cells in culture were identified as olfactory receptor neuronal cells based on morphology and the expression of neuron specific proteins: neuron specific enolase (NSE), microtubule associated protein 2 (MAP2), tau protein and synaptophysin. These olfactory neurons present in the cultures were electrically active and were able to respond to physiological concentrations of odorants as assayed by patch-clamp analysis. The olfactory receptor neurons in culture changed morphologically once they were removed from the tissue. The neurons in culture had many branching processes, unlike olfactory receptor neurons *in vivo* which are bipolar. The authors were able to maintain the cultures for 1-2 weeks in low density or for 25 days in high density. However, when the cells were grown at high density, the non-neuronal cells formed a confluent bed layer under the neurons within 4 days and impeded electrophysiological and morphological observations. Similarly, Chuah *et al.* (1991) developed dissociated cultures of olfactory epithelium that were able to grow at low density in culture in the presence of astrocyte monolayers but they were not able to maintain the cultures for longer than 5 days in culture. Pixley (1992) co-cultured dissaggregated nasal epithelial cells, containing both immature and mature neurons, on monolayers of CNS astrocytes. These cultures were able to survive for more than 35 days and differentiated into OMP$^+$ neurons from neuronal progenitor cells as demonstrated by [^{3}H] thymidine pulse labelling experiments. These experiments demonstrated that astrocyte-derived factors promoted neurogenesis

in this system.

Dissociated cultures have been used to study the effect of growth factors on neurogenesis in the olfactory epithelium (Mahanthappa & Schwarting 1993). This culture system has proven to be very useful in providing much information about growth factor requirement for neurogenesis in the olfactory system. The cells in culture were initially identified by morphology and immunocytochemistry. The two main cell types present were the keratin$^+$ basal cells which were distinguished from the neuronal cells that expressed markers for NCAM, vimentin and the olfactory markers, CC2 and IB2. [^{3}H] Thymidine uptake in conjunction with keratin and NCAM immunocytochemistry revealed that epidermal growth factor (EGF) maintained the basal cell population while transforming growth factor (TGF) β2 appeared to induce keratin$^+$ basal cells to develop into NCAM$^+$ neuronal cells, since NCAM$^+$ clusters of neurons emerged from the periphery of basal cell colonies. However, neither EGF, TGF β2 nor a combination of the two was able to promote neurite survival. Thus the [^{3}H] thymidine uptake experiment showed that the phenotypic change observed in the presence of these growth factors was due to proliferation and neurogenesis rather than survival of the cells. TGF β2 was more effective than TGF β1 in its neurogeneic effect. Furthermore, antibody neutralisation of TGF β2 blocked baseline levels of neurogenesis *in vitro* and thus suggested that TGF β2 was the endogenously produced neurotrophic factor in the olfactory epithelium. Neither nerve growth factor (NGF) nor brain derived neurotrophic factor (BDNF) had any effect on the survival of the olfactory epithelium neurons nor their basal cells. However, in the presence of both BDNF and TGF β2, a subset of olfactory neurons was maintained for 1 week thus showing that in the presence of TGF β2, BDNF provided some degree of neurotrophic support to the cells in culture.

Cells in the olfactory system undergo cell death at a high rate due to exposure to the external environment. There is thus a constant equilibrium between neurogenesis and cell death. The effect of cell death in the olfactory system was studied by Holcomb *et al.* (1995) using dissociated cultures. This *in vitro* system provided evidence that cell death occurred through a process of apoptosis. Apoptosis regulates neuronal number in the olfactory epithelium at multiple stages in the neuronal lineage. Results showed that apoptosis occurred in all neurons and their precursors at different stages of development but not in non-neuronal cells. Holcomb *et al.* (1995) were also able to inhibit apoptosis with various growth factors, such as BDNF, neurotrophic factor (NT)3, NT5 as well as aurintricarboxylic acid, which was also found to be the most effective. They showed that an increase in apoptosis i*n vitro* was preceded by an increase in phase-bright neurite extending cells.

As an alternative to continuously generating dissociated cultures, immortalised cell lines can be generated. Cell lines are a consistent and reliable source of material for long term, repeated or related experiments since the immortalised phenotype can be maintained over numerous passages and can be used for genetic manipulations. The cell lines are clones and so permit the properties of the cells to be examined with or without the influence of the other cell types present in the olfactory epithelium. As a single cell type, the effect of various factors can be directly studied on this clone. In addition, the response of other cells in the olfactory system towards this cloned cell can be determined by culturing these cloned cells on feeder layers containing other olfactory cells or by transplanting this cloned cell back into the host animal. Researchers have used a number of methods in an attempt to immortalise olfactory neurons. Coon *et al.* (1989) established spontaneously immortalised cultures by serial dilution and cloning. These cells, which migrated away from explant tissue of olfactory epithelium survived for 2.5 years and could thus be said to constitute a spontaneously immortalised cell line. Although incompletely differentiated, these cells qualified as olfactory neuronal cells since they responded to odorants by an increase in cAMP - a component of the second messenger system involved in the transduction of an odorant stimulus into a neural signal.

Goldstein *et al.* (1997) subsequently showed that the cells from the Coon cell line (NIC cells) expressed antigens characteristic of several olfactory epithelial cell types when maintained in complete medium containing serum and tissue extracts. The NIC cells expressed GBC-1/ GBC-2 (markers characteristic of globose basal cells in normal and regenerating olfactory epithelium), vimentin (expressed by olfactory receptor neurons), HB/5-1 (a marker for horizontal basal cells and sustentacular cells), sus-5 and cytokeratin 18 (sustentacular cell markers). They were negative for glial fibrillary associated protein (GFAP), NCAM or GAP43. When the cells were grown in basal medium without serum or tissue extract, the NIC cells stopped proliferating (BrdU-negative) and differentiated towards a neuronal phenotype that expressed neurotubulin and NCAM. In basal medium the NIC cells only remained viable for a short period of time after which the cell numbers decreased and the cells died by apoptosis. Basic fibroblast growth factor (bFGF) prevented its differentiation by maintaining them in an early stage of neurogenesis. The authors proposed that bFGF, which is found in both mature olfactory sensory neurons and sustentacular cells and increased after cell damage, is important for cell regeneration.

Satoh & Takeuchi (1995) established a horizontal basal cell line, DBC1.2 by spontaneous immortalisation of explanted embryonic murine E14.5 olfactory epithelium. Cells from this cell line expressed keratin but lacked NCAM expression and were thus regarded as horizontal basal cells.

Western blotting analysis revealed that the DBC1.2 cells were induced by TGFβ or a high concentration of calcium to express the 120 kDa and the 140 kDa isoforms of NCAM. The 180 kDa isoform was not detected with these treatments. These cells could thus be regarded as capable of differentiating along the neuronal pathway in the presence of TGFβ or an increased concentration of calcium. The authors interpreted the induction of NCAM expression as evidence for a lineage relationship between keratin$^+$ cells (horizontal basal cells) and olfactory receptor neurons.

Others have attempted to immortalise olfactory neurons with proto-oncogenes. Calof & Guevara (1993) tried to immortalise olfactory neuronal precursor cells with *c-myc*, but the cell lines that emerged had the characteristics of the ensheathing or Schwann cells of the olfactory nerve. Their OEmyc790 cells morphologically resembled primary olfactory ensheathing cells or Schwann cells and expressed GFAP and S100 (general markers for non-myelinating Schwann cells *in vitro* and *in vivo*) but none of the markers characteristic of olfactory receptor neurons (NCAM, olfactory receptor neuron gene products or vimentin) by immunochemical analysis. The authors suggested that the olfactory ensheathing progenitor cells originated in the *in vitro* cell population that consisted of keratin$^+$ basal cells and that the olfactory ensheathing cell lineage was separate from the neurogenic lineage of olfactory receptor neurons.

MacDonald *et al.* (1996) were more successful. Using N-myc transfer they immortalised olfactory precursor cells to obtain cell lines which could be divided into three classes, that the authors suggested represented different stages in development along the lineage pathway. Based on their morphology and phenotypes, the cell lines were classified as horizontal basal cells, as globose basal cells or as glial precursor cells. Some of the globose basal cells expressed the transcript for OMP, a marker of mature cells, so providing evidence that precursor cells may differentiate in culture. Immunocytochemistry, however, failed to demonstrate expression of mature OMP protein.

Largent *et al.* (1993) and Servenius *et al.* (1994) attempted to immortalise olfactory receptor neurons from transgenic mice. They targeted the expression of the SV40 large T antigen (SV40 Tag) to the mature olfactory receptor neuron flanking sequences from the OMP, which is expressed only in mature olfactory receptor neurons.

Largent *et al.* (1993) reported a single mouse cell line transgenic for the SV40 Tag under the control of 3 kb of rat OMP promoter region. The transgenic animal presented with hypoplastic olfactory mucosa and developed olfactory tumours at a low frequency (3% of the hemizygous and 7% of the homozygous by 1 year). The cells derived from these tumours did not express neuronal

characteristics of fully differentiated olfactory receptor neurons despite the fact that SV40 Tag expression appeared to be confined to olfactory receptor neurons *in vivo* in the transgenic founder animal.

Servenius *et al.* (1994) used a larger construct that encoded the SV40 Tag under the control of a 6 kb mouse OMP promoter region. The transgenic mice showed a higher incidence of tumours that had a different distribution than those of Largent *et al.* They subsequently isolated, from the tumour bearing mice, five neuroblastoma cell lines that expressed endogenous OMP as determined by immunostaining and northern blot analysis. The cell lines also expressed light, medium and heavy neurofilaments, NSE and synaptophysin - all antigenic markers for cells of neural derivation. Although OMP was originally considered to be a specific marker for functionally mature olfactory receptors, the transgenic mice also developed tumours in their adrenal glands and sympathetic ganglia. The authors proposed that OMP is not unique to the olfactory system and that it is involved in the development of the sympathetic nervous system with possibly, a function at discrete stages of differentiation. Low levels of OMP expression have been found in the cerebellum, hypothalamus and the spinal cord (Baker *et al.* 1989).

The olfactory system provides an attractive experimental substrate for the study of neurogenesis - a field of investigation that would benefit greatly from the availability of immortalised, clonal precursor cell types which resemble and behave like their *in vivo* counterpart. In this study I report my attempts to immortalise olfactory neuronal precursor cells using the SV40 retroviral vector carrying a temperature sensitive mutation in the SV40 Tag. If successful I felt I should be able to maintain proliferating populations of cells for indefinite periods at the permissive temperature for SV40 Tag expression. At the non-permissive temperature, the SV40 Tag would be inactivated and the cells could be followed as they reverted to normal cellular behaviour and entered a differentiation pathway.

The experiments that I describe later in this thesis involved the use of an SV40/retrovirus shuttle vector to transform olfactory epithelial cells. This vector contained transforming elements derived from SV40 and the Moloney murine leukaemia virus (M-MLV). I feel it appropriate to review, briefly, the relevant information regarding these two oncogenic viruses.

1.6 SV40: The DNA virus

Simian virus 40 (SV40) is a small, double stranded (ds) DNA virus that was first isolated by Sweet and Hilleman (1960) from Rhesus monkey kidney cells. It has the ability to induce tumours in certain animals (Tooze 1980). SV40 is a member of the papovavirus family (Howley 1980). The family name is derived from three of its members: the **pa**pilloma virus, **po**lyoma virus, and the SV40 that was originally called the **va**cuolating virus because of its ability to induce multiple vacuoles in the cytoplasm of African green monkey kidney cells.

The circular 5243 bp SV40 genome contains a promoter-enhancer element of approximately 300 bp that divides the genome into an early and a late region. Early studies with the SV40 virus showed that the early region of the genome encodes two tumour-associated antigens: the large T antigen and the small t antigen. The early genes are transcribed both in SV40 transformed cells and during the early stages of the SV40 lytic infection. The early gene primary transcript undergoes differential splicing to produce the mRNA for the small t and large T antigens. The smaller mRNA codes for the large T antigen and the larger mRNA codes for the small t antigen (Berk & Sharp 1978; Ziff 1980).

The small t antigen is not essential for transformation, as SV40 deletion mutants defective for this protein are still capable of transforming cells. It does, however, enhance the activity of the large T antigen (Montano *et al.* 1990) that is necessary for transformation (Bikel *et al.* 1988; Brugge & Butel 1975; Osborn & Weber 1975; Tegtmeyer 1975).

1.6.1 *The transforming activity of the SV40 Tag*

The SV40 protein is found predominantly in the nucleus of transformed cells and has been shown to be endowed with many functions. These include the regulation of SV40 replication, the regulation of host cell gene expression and transformation of cells by association with such cellular proteins as p53, pRb, p107, p300, heat shock protein p73, AP2 and DNA polymerase α.

The transforming activity of SV40 Tag has been the subject of an extensive recent review by Manfredi and Prives (1994) and can be summarised briefly as follows:

The SV40 Tag contain four domains (labelled A-D) that function in the following manner.

- Region A, comprising 82 amino-acid residues, binds to DNA polymerase α (Gannon & Lane 1987) and appears to be involved in the transcription activation function of the SV40 Tag. The cellular heat shock protein, hsp73 also binds to this region (Sawai & Butel 1989) as does the cellular protein p300 that binds in the hypophosphorylated and ubiquitinated form (Avantaggiati *et al.* 1996).
- Region B, a 17-residue domain adjacent to A, associates with pRb (Lane & Crawford 1979) and the pRb-related molecule, p107 (Dyson *et al.* 1989).
- Region C, is a 12-residue region that binds a 'nuclear localisation signal".
- Region D spans two subdomains. The first includes a 99 amino acid stretch that, with region A, binds to DNA polymerase α (Gannon & Lane 1987). The second consists of a 93 stretch region that, with the 99 residue segment, binds p53.

1.7 An engineered retrovirus that carries the SV40 Tag

Although primary cultures have been used to study neuronal differentiation, they do have a number of drawbacks:

- they have a limited lifespan
- they are non-renewable as fresh animals have to sacrificed for each study
- a large number of animals are required for each experiment
- they consist of a heterogeneous cell populations.

One way around these problems is to establish clonal cells immortalised with oncogenes. A cell line with a stable phenotype, that could be induced to differentiate along a particular pathway, would be a valuable biochemical tool. In this thesis, I describe how I established clonal cell lines from embryonic olfactory neuroepithlium infected with a retrovirus carrying the SV40 Tag with temperature sensitive mutations U19 and tsA58. Details of the procedure that I used are described in Chapter 2. At this point, however, it is appropriate to review the history of the virus.

In 1984, Cepko, Roberts and Mulligan developed a murine retrovirus shuttle vector system for introducting DNA into mammalian cells. This they did by combining the transcription units derived from an integrated Moloney murine leukaemia provirus (Hoffman *et al.* 1982) with the pBR322 sequence necessary for the propagation of vector DNA in *E. coli*. The M-MLV sequence retained in the vector included the long terminal repeats (LTRs) that are necessary for integration, the initiation of viral transcription and the polyadenylation of viral transcripts; the sequences necessary for reverse transcription of the viral genome and for the encapsidation of viral RNA. The retroviral

sequence encoding *gag, pol* and *env* proteins were removed and replaced with two restriction enzyme sites (Bam HI and Xho I sites) that would allow introduction of foreign DNA for transfection.

DNA sequences encoding G418 resistance in mammalian cells (kanamycin resistance in *E. coli*) were obtained from transposon Tn5 and these together with sequences encoding the SV40 and the pBR322 origins were introduced into the Xho I site to allow for selection of mammalian cells harbouring the provirus and for rapid recovery of free or integrated proviral genomes as bacterial clones. A diagram of the vector pZip-Neo SV(X)1 is shown in Fig 1.3.

Almazan and McKay (1992) introduced, into the Bam HI site of the pZip-Neo SV(X)1, a DNA fragment encoding the SV40 Tag that contained the temperature sensitive mutation, A58 (Tegtmeyer 1975) at nucleotide 3504 and the U19 mutations (Paucha *et al.* 1986) at nucleotides 4345 and 4364. The SV40 Tag was known to be capable of establishing immortal cell lines when expressed in embryonic cells (Jat & Sharp 1986; Petit *et al.* 1983). Jat & Sharp (1986) showed that the introduction of coding sequences for SV40 Tag that contained the U19 mutation gave a much higher frequency of immortalisation without crises than was observed with the wild type SV40 Tag. This higher efficiency of immortalisation was useful since the target cells for transfection in embryonic epithelium are scarce.

As indicated earlier, immortalisation could be expected to result in the establishment of a line of cells that were fixed at a definite stage of differentiation and would divide indefinitely under the influence of the SV40 Tag. It was necessary to have some means of manipulating these cells so that they would proceed from that point to differentiating further by inactivating the SV40 Tag. To achieve this a fragment containing the temperature sensitive A58 mutation (obtained earlier by Tegtmeyer (1975) by chemical mutagenesis of the wild type SV40 virus) was introduced into the SV40 U19 large T antigen gene so that the cells grown at the permissive temperature of 33°C would proliferate without differentiation. When, however, the cultures were shifted to the non-permissive temperature of 39°C (the normal body temperature of the mouse) the SV40 Tag protein is no longer functional and differentiation would proceed.

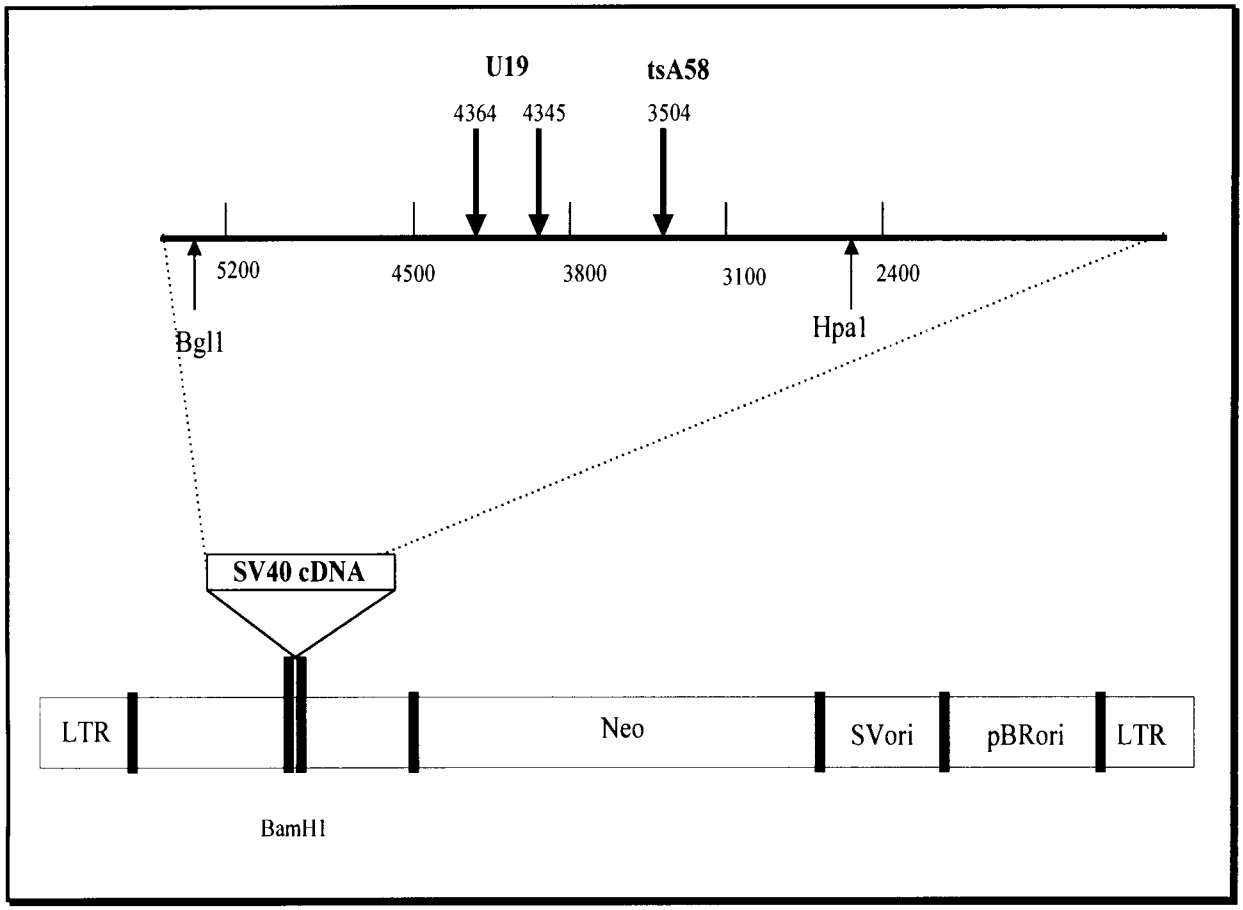


Fig 1.3 Construction of the plasmid SV40U19tsA58. The BglI-HpaI DNA fragment of the SV40 Tag was inserted into the BamHI site of the pZip Neo SV(X)1 shuttle vector in the sense orientation with respect to retroviral transcription driven by the cis-acting transcriptional regulatory sequences in the left-hand long terminal repeats (LTRs). The tsA58 mutation was on nucleotide 3504 and the U19 on the nucleotides 4345 and 4364. [Modified from Almazan and McKay (1992)]

Cells transduced with the vector encoding the defective retroviral construct, although transformed, would not produce infective virus that could be used to immortalise other cells. To overcome this problem a cell line was required that would efficiently complement the replication - defective, packagable retroviral RNA by providing, in trans, the products necessary for virion production. Mann, Mulligan and Baltimore, in 1983 produced such a cell line (ψ2). By transforming mouse fibroblast (NIH-3T3) with a retrovirus packaging mutant pMOV-ψ^-. Transfection of these cells with pZip-Neo SV(X)1 released infectious virus into the supernatant which could be used for transforming other cells.

I received from Dr R. McKay a culture of ψ2 cells that produced infectious pZip-Neo SV(X)1 virus.

1.7.1 *Molecules which interact with the SV40 Tag and that are implicated in its transforming activity*

In this system the infected embryonic cells integrate the retroviral sequences into the genome and, at the permissive temperature, large amounts of mutant SV40 Tag are produced under the control of regulator elements of the M-MLV LTRs. The SV40 Tag is believed to associate with a number of proteins that are involved in the control of the cell cycle and which function as products of 'tumour repressor genes' *in vivo*. These include p53 (Lane & Crawford 1979), pRb (DeCaprio *et al.* 1988), p107 (Dyson *et al.* 1989; Ewen *et al.* 1991), p130 (Hannon *et al.* 1993), p300 (Avantaggiati *et al.* 1996), hsp p73 (Sawai & Butel 1989) and DNA polymerase α (Gannon & Lane 1987).

1.7.1.1 p53

The SV40 Tag is thought to induce transformation by interacting with key cellular proteins that normally function to regulate cell proliferation. One such protein is p53, which is found in a physical complex with SV40 Tag in transformed cells. Because p53 blocks the growth or transformation of cells in culture and is lost in tumours, it is considered to be a "tumour suppressor protein". The wild type p53 is a nuclear phosphoprotein that binds DNA in a sequence specific manner and activates transcription either by acting as a transcription factor itself or by activating other transcription factors (Manfredi & Prives 1994). This sequence-specific transcriptional activity appears to be essential for its role as a tumour suppressor. A number of genes involved in the control of the cell growth are known to be transcribed in a p53-dependent manner, including the *WAF1/CIP1* gene (Picksley & Lane 1994). Although p53 acts as a transcriptional activator of genes containing p53-binding sites, it is also capable of strongly inhibiting transcription from many other genes lacking p53-binding sites (Ko & Prives 1996; Manfredi & Prives 1994). p53 acts as a tumour suppressor protein by indirectly causing hypophosphorylation of Retinoblastoma (pRb) and inactivating the cells in the G_1/S phase prior to the onset of DNA synthesis (Manfredi & Prives 1994; Picksley & Lane 1994). For this function as well as for the function of inducing apoptosis in response to DNA damage, p53 is often regarded as the "guardian of the genome" (Lee *et al.* 1994).

In 1979, Lane and Crawford identified a 53K product (p53) that co-precipitated with the SV40 Tag when they immunoprecipitated extracts of the SV40 transformed mouse cell line with antiserum raised against purified SV40 Tag. The p53 protein has been shown to be essential for tumorigenesis induced by SV40 Tag. However, the use of mutations of p53 (Schmieg & Simmons 1993) and

mutants of the SV40 Tag (Chen *et al.* 1992) clearly showed that the wild type p53 is not sufficient for transformation but that other proteins are also required for full transformation of cells.

SV40 Tag binds to p53 and inhibits the ability of p53 to bind to DNA and likewise inhibits its ability to activate a promoter containing a p53 binding site (Manfredi & Prives 1994).

The SV40 Tag appears to stabilise p53 and thereby increase its steady state levels. Wild type p53 is highly unstable in cells with a half-life of the order of 20 minutes (Oren *et al.* 1981). In the presence of SV40 Tag, p53 is stabilised such that its half-life becomes of the order of several hours (Reihsaus *et al.* 1990) and this stabilisation appears to occur post translationally (Manfredi & Prives 1994; Reich *et al.* 1983). In the SV40 Tag transformed cells, p53 is more heavily phosphorylated than in the absence of the SV40 Tag. Since transformation-defective mutants of the SV40 Tag lack the ability to stimulate phosphorylation of p53, it is proposed that the SV40 Tag induces or activates a protein kinase that phosphorylates p53 and this phosphorylation is necessary for SV40 Tag mediated transformation (Scheidtmann & Haber 1990).

1.7.1.2 Retinoblastoma protein (pRb) and pRb related family proteins: p103 and p107

Retinoblastoma is a malignant tumour of the retina that occurs either in hereditary or sporadic form. Two mutations involving each of the two copies of the retinoblastoma gene result in retinoblastoma. The retinoblastoma gene (Rb) is regarded as a "tumour suppressor gene", since inactivation of both alleles results in uncontrolled tumour proliferation.

The retinoblastoma gene encodes a nuclear phosphoprotein ($pp11^{RB}$) also designated pRb that forms a stable complex with SV40 Tag (DeCaprio *et al.* 1988). The authors immunoprecipitated extracts from the CV-IP monkey cell line that synthesised the SV40 Tag with monoclonal antibodies to either pRb or to the SV40 Tag. They found that both proteins can be co-precipitated with either antibody. They also identified a small co-linear sequence (residues 105-114) of the SV40 Tag that is essential for pRb binding.

The pRb-binding site on the SV40 Tag contains the motif LXCXE (single letter amino acids) (Manfredi & Prives 1994) that is important for binding to the A/B pocket of the pRb (Knudsen & Wang 1996). SV40 Tag only binds to the active, hypophosphorylated form of pRb. In this form, pRb exerts a negative growth effect on the cells. Upon phosphorylation of pRb by cyclin-dependent

kinases (Cdk) complexes (cyclin-Cdk complexes) cells are allowed to proceed into the S-phase. The SV40 Tag can also stimulate phosphorylation of pRb in quiescent cells. Thus SV40 can inhibit pRb function by both direct binding and by stimulating phosphorylation (Picksley & Lane 1994).

The pRb protein exerts its control over cell proliferation by association with a variety of cellular proteins, most notably the E2F family of transcription factors. E2F binding sites are present in a variety of genes involved in initiating DNA synthesis (Picksley & Lane 1994). Association of pRb with E2F protein prevents the transcriptional activation of a variety of genes, the products of which are central to the onset of the DNA synthesis cycle (Hinds 1995). The E2F family of transcription factors do not contain this LXCXE motif but also bind to the A/B pocket that is now referred to as the 'large A/B pocket" because the binding of the E2F includes the C-terminal amino acids (Knudsen & Wang 1996).

Progression of eukaryotic cells through the cell cycle is a complex process that is regulated by both external and internal checkpoints. In untransformed cells, p53 and pRb interact with each other as well as other proteins to regulate the cell cycle. Under conditions that threaten the genetic integrity of the genome, p53 accumulates in a transcriptionally active form and triggers transcription from the *WAF1/CIP1* gene that encodes p21, a Cdk inhibitor. This accumulates in the cell nucleus, titrates out the activity of the critical Cdk/cyclin complexes (Michieli *et al.* 1994) and eventually blocks cell cycle progression. This enforces a growth arrest in the G_1 phase. High levels of p21 are believed to maintain pRb in a constitutively active state by blocking hyperphophorylation of pRb family proteins (Haffner & Oren 1995). In the hypophosphorylated form pRb is able to bind to the E2F family of transcription factors so inhibiting their transcriptional. The hyperphosphorylation of pRb is only permitted by p53 if the cell is ready to divide (Picksley & Lane 1994). A dual signal model has been proposed for p53. On the one hand p53 induces expression of p21 and causes cell cycle arrest; alternatively, p53 can activate the apoptotic pathway (El-Diery *et al.* 1994). The decision fork depends on the presence of functionally active pRb in the cell (Haffner & Oren 1995). DNA damage, due to DNA breaks induced directly or during attempts to repair or replicate damaged DNA, causes activation of active p53 which appropriately corrects this by inducing G_1 arrest in the presence of pRb or inducing apoptosis in the absence of pRb (Haffner & Oren 1995; Picksley & Lane 1994). The SV40 Tag prevents apoptosis by binding to the active form of p53 (Shen & Shenk 1995).

The interaction of pRb with SV40 Tag is essential for the transformating activity of the latter. Recently, a number of pRb-related cellular proteins have been identified and shown to bind to SV40

Tag. These proteins, including p107 and p130, are classified as members of the pRb family of "pocket" proteins (Kaelin *et al.* 1991).

It is suggested that pRb and p107 act at different stages of the cell cycle; pRb complexes with E2F in the G_1 phase in primary and in established human cells and p107 complexes with E2F in the S-phase of these cells (Shirodkar *et al.* 1992). The SV40 Tag also binds to the unphosporylated form of p107 and has the ability to disrupt both these complexes (Dyson *et al.* 1989).

By using SV40 Tag mutants defective in pRb binding, Chen *et al.* (1992) showed that the SV40 Tag/p53 complex was not sufficient for tumour formation. However, they showed that pRb/p107 complex was essential for tumorigenesis in the test tissue, choroid plexus. Whether pRb alone is sufficient for tumorigenesis of the choroid plexus was not answered in this article.

The difference between the roles of pRb and other family members in linking cell cycle control to various aspects of growth control is not understood. However, it is speculated that pRb family of proteins might interact with a different subset of transcription factors and in that way regulate cell cycle control (Hannon *et al.* 1993).

1.7.1.3 p300 transcriptional co-activator

The phosphoprotein p300 is likely to be involved in the control of cell growth. The protein p300 is a transcriptional co-activator whose function is presumably inhibited upon binding to SV40 Tag by forming a specific complex with it (Avantaggiati *et al.* 1996). The authors suggest that these complexes act as repressors of transcription of critical genes. The authors showed in various cell lines that the SV40 Tag co-precipitated with p300 protein which was hypophosphorylated but ubiquitinilated.

The interaction of SV40 Tag with p53 and pRb is important for its transforming function. The role of the interaction of SV40 Tag with other cellular proteins such as p107, p103 and p300 is as yet undefined.

1.8 The use of SV40 in producing cell lines

The recombinant retrovirus carrying the SV40 Tag has been successfully used to establish many cell lines (Table 1.1).

Retroviral insertion only takes place in mitotically active cells and not in cells that are terminally differentiated. This limits the cell population that can be immortalised using this technique. If a more mature cell type is required as an immortal cell line transgenic mice are used.

Table 1.1 Immortalisation of cell lines with the SV40 retrovirus

Retrovirus	Cell line derived from	Reference
Wild type SV40	Mandibular condyle cells	Bhalerao *et al.* (1995)
	Leydig cells	Nagpal *et al.* (1994)
Temperature sensitive A58 SV40 mutant	Precursor cells from the CNS	Fredriksen *et al.* (1988)
	Rat embryonic fibroblast cells (REF)	Jat & Sharp (1989)
	Embryonic hippocampal cells	Mehler *et al.* (1993)
	Embryonic hippocampal cells (HiB5)	Renfranz *et al.* (1991)
	Raphe cells	Stringer *et al.* (1994)
	Medullary raphe cells (RN33B)	Wittemore & White (1993)
U19 SV40 mutant	Rat embryo fibroblast cells (REF)	Jat & Sharp (1986)
Transgenic mice - SV40	Fibroblast cells (H-2K)	Jat *et al.* (1991)
	Hypothalamus cells (GT1)	Mellon *et al.* (1990)
	Olfactory bulb cells	Servenius *et al.* (1994)
	Kidney tubule cells (TKC2)	Taher *et al.* (1995)

A transgenic mouse is produced by transferring a gene of interest into a fertilised oocyte. This is then reimplanted into the uterus of a female foster mother. The tissue that is selected for tumorigenesis is determined by co-transferring the appropriate promoter and enhancer elements.

A number of researchers have successfully isolated cell lines from such transgenic mice expressing either wild type SV40 (Mellon *et al.* 1990; Servenius *et al.* 1994) or the temperature sensitive SV40 mutant (Jat *et al.* 1991; Robinson *et al.* 1994; Taher *et al.* 1995).

Jat *et al.* (1991) produced transgenic mice by using the H-2k^b gene promoter (which is expressed by most cells but the transcription of the H-2k^b gene can be increased by γ-interferon) to control temperature sensitive SV40 Tag gene expression. Immortalisation of the cell type of interest was achieved by removing tissue from transgenic mouse and incubating the cells at 33°C in the presence of γ-interferon. Fibroblast cultures (H-2K) were established from these animals. Results from these cell lines showed that H-2K cell line was only able to proliferate at 33°C in the presence of γ-interferon but not at 39°C. *In vitro*, however, the SV40 Tag was not completely inactivated and, at 39°C (murine body temperature), lethal thalamic tumours developed.

Taher *et al.* (1995) showed that the TKC 2 cell line immortalised from the kidney of transgenic mice expressing temperature sensitive SV40 Tag gene proliferated well at 33°C but, when shifted to 39°C, died by apoptosis. When temperature sensitive cells were shifted to 39°C, the SV40 Tag antigen was inactivated. This led to the expression of p53 which has been shown to induce apoptosis (El-Deiry *et al.* 1994).

Several immortalised GnRH expressing cell lines have been generated by targeted tumorigenesis (Mellon *et al.*1990; Radovick *et al.* 1991). By targeting the expression of the SV40 Tag to the GnRH neurons with the GnRH gene regulatory sequence resulted in the development of anterior hypothalamic tumours (Mellon *et al.*1990) or olfactory tumours (Radovick *et al.*1991) in the transgenic mice. The tumours were taken and cultured to generate the GT1-1/3/7 neuronal cell lines (Mellon *et al.* 1990) or NLT and Gn11 cells (Radovick *et al.*1991) respectively. As a control cell line in our studies we used GT1-7 cell line that express GnRH. These cells have a neuronal morphology in culture, they express neuronal markers, GnRH mRNA; they contain GnRH immunoreactive material; and they secrete GnRH into the medium in response to depolarisation.

The NLT and Gn11 cell lines were interesting in that both were derived from the same olfactory tumour but expressed different concentrations of GnRH as determined by radio-immunoassay (RIA). NLT cells secreted 10x higher levels of GnRH than the Gn11 cells. Results with RT-PCR and RNase protection assays showed that the lower GnRH secreting capacity found in the Gn11 cells was due in part to a splice variant lacking exon 2 of the proGnRH gene, which encoded the GnRH decapeptide. The same spliced variant and the mature proGnRH were also expressed in the olfactory area and the preoptic area-anterior hypothalamus but only the mature proGnRH was detected in the hypothalamus. The prevalent transcript in the olfactory area was the mature proGnRH transcript (Zhen *et al.* 1997).

1.8.1 *The use of SV40 Tag in the establishment of olfactory epithelial cell lines.*

Since I was interested in immortalising precursor cells or stem cells from the olfactory epithelium in the hope of inducing them to differentiate into either olfactory receptor neurons or GnRH neurons, I immortalised primary cultures from olfactory epithelium with the *pZipNeoSV40U-19tsA58* retrovirus.

1.9 Aims of the project

Research on cell lines has greatly improved our understanding of many important biological questions. Recently various techniques have been developed to immortalise neural cell lines. I will attempt to establish an olfactory progenitor cell line by introducing an immortalising gene, the temperature sensitive SV40, into the primary cultures derived from the embryonic mouse olfactory neuroepithelium and thereby establish an *in vitro* tool for the olfactory system. These conditionally immortalised cell lines will be fully characterised to determine its origin and its properties in culture. If successful in immortalising olfactory stem cells or progenitor cells from the olfactory epithelium, I hope to induce them to differentiate and see whether olfactory neurogenesis can be mimicked *in vitro*.

These olfactory cell lines would provide an *in vitro* system which would allow the study of the mechanisms directing neuronal differentiation. Retinoic acid will be used to set up an experimental system whereby the effects of various factors on growth and development of the cells can be studied at the permissive temperature of 33°C and when shifted to the non-permissive temperature of 39°C. With the increasing availability and knowledge of growth factors and in particular, neurotrophic factors, these cell lines could be used as a tool to study the mechanism of neuronal differentiation with various neurotrophic factors in the olfactory system.

Chapter 2

Methods and Materials

2.1 Animals

Mice used in this study were obtained from the UCT Animal House, Medical School, Observatory. Primary cultures of olfactory neuroepithelium were prepared from E10.5 C3H mouse embyros (Cattanach *et al.* 1977). Females of mating pairs were examined daily and the morning a vaginal plug was detected was designated as day 0.5 of pregnancy. Postnatal brains were taken from one day old mice pups for immunocytochemistry of primary cultures.

2.2 Tissue culture techniques

Unless otherwise stated the cells were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose) containing 3.7 grams/litre $NaHCO_3$ and 10% heat inactivated (58°C; 30 minutes) fetal calf serum (FCS) - hereafter referred to as DM-10. Cultures were routinely maintained at the permissive temperature of 33°C in a humidified atmosphere of 10% CO_2. For experimental purposes at the non-permissive temperature, cultures were incubated at 39°C in a humidified atmosphere of 10% CO_2. Cultures were routinely maintained in the absence of antibiotics. Only when cultures were plated out for experiments were antibiotics used; either gentamycin (50 mg/ml) at 50 μg/ml or penicillin (10 000 U/ml) at 100 U/ml and streptomycin (10 mg/ml) at 100 μg/ml.

Phosphate buffered saline (PBS) containing trypsin (0.25%) and EDTA (0.02%) was added to the washed monolayer of cells for 1 minute at room temperature (RT) to harvest cells at 33°C. The cells were shaken to disaggregate them and then resuspended in DM-10. Cells were pelleted at 1000g for 5 minutes. Harvesting of the cultures maintained at 39°C required more gentle techniques since they were more fragile and trypsin caused the cells to degrade. Cell monolayers were washed in PBS, incubated in 5 ml of PBS and released with a cell scraper. The cells were pelleted as for cells at 33°C.

2.2.1 *Primary cultures*

Embryos were dissected in ice-cold calcium- and magnesium- free Hank's balanced salt solutions (CMF-HBSS) on ice. Olfactory placodes from three embryos were surgically excised cleaned from surrounding tissue and placed in 0.25 ml Hanks balanced salt solution (HBSS) containing 0.04% trypsin in PBS (pH 7.5) for 10 minutes at RT after which disaggregation was terminated by adding

1.5 ml DM-10. The dissociated cells were washed, resuspended in DM-10 and plated on 35 mm culture dishes previously treated as follows: 0.75 ml of a solution of polyornithine (15μg/ml) was added to each dish and these were incubated for 30 minutes at 33°C. The residual polyornithine solution was aspirated and the dishes were washed thrice in PBS solution and then incubated in PBS till required.

Primary cultures of mouse brain cells (a mixed population of cells) served as positive controls for GFAP, O1, Keratin, NF160, nestin and vimentin immunocytochemical staining since these primary cultures display all the antigens of interest. These cultures were prepared as follows: (Turner & Bachelard, 1987; Conn 1990) neonates were dipped in 95% ethanol and decapitated with sharp scissors. The head was placed in ice-cold CMF-HBSS, freed of meninges and all fatty tissue, and transferred to 1 ml 0.04 % trypsin in PBS. The tissue was dissociated over 10 minutes by pipetting sequentially at RT with a 10-, 5- and 1- ml pipettes to obtain a single cell suspension. The suspension was centrifuged at 1000g for 5 minutes and cells were resuspended in DM-10 containing Gentamycin 50 μg/ml. The resulting cell suspension was then plated as 100 μl drops on 13 mm round, polyornithine-coated Thermonox coverslips, set in the wells of a 24-well tissue culture (TC) dish. The cells were maintained for one week and then fixed with 4% (wt/vol) Paraformaldehyde (PFA) in PBS for 30 minutes at 4°C and then examined immunocytochemically as described below.

2.2.2 *Preparation of retrovirus*

The producer cell line, ψ2 (Almazan & McKay 1992) (a gift from R.D.G. McKay at the National Institute of Neurological Disorders, NIH, Bethasda), was maintained at 33°C in DM-10 (Price 1993). To prepare retrovirus supernatant, medium covering 80% confluent cultures was replaced with fresh medium that was collected sixteen hours later. This was centrifuged (1000g; RT; four minutes), filtered through a 0.45 μm filter and stored in 10 ml volumes at -70°C.

The titre of this retrovirus was determined by preparing ten-fold dilutions (10^0 to 10^{-7}) of virus-containing supernatant. These were used to infect semi-confluent cultures of 3T3 cells by adding 2 ml of each dilution, adjusted to contain 8 μg/ml polybrene, to each 35 mm dish and incubating for 24 hours at 33°C. The medium was then replaced with DM-10 and the cultures were incubated for a further 48 hours. Neomycin-resistant colonies were selected by replacing the medium with DM-10 containing 1 mg/ml of Genetecin (G418). Selective medium was renewed every 2 days and resistant colonies appeared after approximately 10 days. These were counted to give a titre of 10^4 cfu/ml.

2.2.3 *Viral infection of primary cultures from the olfactory epithelium*

Primary cultures of E10.5 mouse olfactory neuroepithelial cells (section 2.3.1), were incubated for 2 hours at 33°C with viral supernatant (10^4 cfu/ml) containing 8 μg/ml polybrene. Two days later the culture medium was replaced with DM-10 containing G418 at 200 μg/ml. The selective medium was replaced every 2-3 days. Within 3-4 weeks G418-resistant colonies were observed. Colonies were picked with cloning rings and expanded into 96-well plates. The clones were subsequently grown at 33°C in DM-10 and expanded up through 35 mm dishes. Sixty different colonies were selected for analysis. These are identified in subsequent sections by the letters 'OP' (for olfactory placode) followed by the number of the clone chosen.

The OP cell lines grew as adherent monolayers in DM-10. Cultures were maintained at the permissive temperature of 33°C and the medium changed every 3-4 days. Cells were passaged at ~80% confluency by releasing with trypsin/ EDTA as described and resuspending in DM-10 for counting and replating or freezing. The passage number was recorded and all experiments were performed below a passage number of 10.

Cells were counted on a standard heamocytometer stage (Morgan & Darling 1993) after adding, to the suspension an equal volume of 0.4% solution of Trypan blue in PBS. They were replated at $1x10^4$ cells/cm^2 in 250 ml TC-flasks.

For storage, cells were frozen in liquid nitrogen at $1x10^5$ cells/ml in DM-10, Gentamycin and 10% dimethyl sulphoxide (DMSO), in 1 ml cryotubes. When required, the frozen cells were thawed rapidly at 37°C. DM-10 was added to the cells to a final volume of 10 mls and then centrifuged at 1000g for 5 minutes. The cell pellets were resuspended in fresh DM-10 and plated into 50 ml TC flasks (Turner & Bachelard 1987).

2.2.4 *Mycoplasma testing*

The OP cells were routinely checked for the presence of mycoplasma by plating and culturing on glass coverslips. When 60-70% confluent the cells were rinsed, fixed with methanol:acetic acid :: 3:1 for 10 minutes at RT, rinsed with PBS, air-dried and stained with Hoescht stain for 1 minute 20 seconds. Stained monolayers were examined with a Nikon fluorescent microscope using a DM400 filter (Freshney 1992).

2.2.5 *Growth curves of OP cell lines*

Growth curves were defined by plating cells at $1x10^4$ cells/cm^2 in 35 mm dishes and incubating at 33°C or 39°C for 21 days with a medium change every 3-4 days. For the 39°C experiments, cells were allowed to settle at 33°C overnight before shifting to 39°C. In the reversion experiment, cells were maintained at 39°C for 8 days, then shifted back to 33°C for a further 13 days. Cells were released from duplicate dishes for each time point and counted after concentrating by centrifugation and resuspension. Trypan blue was added to all suspensions to assess viability.

Doubling times were calculated from the linear region of a semi-log plot of the mean duplicate cell counts as a function of time.

2.2.6 *Retinoic acid experiment*

2.2.6.1 Growth conditions in tissue culture

To induce differentiation of OP cell lines at 39°C, the cells were plated out at $1x10^4$ cells/cm^2 on Thermonox coverslips in DM-10 and allowed to settle at 33°C overnight. Once settled, the medium was changed to a defined N2 medium (Barnes & Sato 1980; Bottenstein & Sato 1979 and Ray *et al.* 1993) (1:1 mixture of DMEM/F-12 containing 5 µg/ml insulin, 100 µg/ml transferrin, 100 µM putrescein, 20 nM progesterone and 30 nM sodium selenite) containing 50 µg/ml Gentamycin, 2% FCS and 10^{-6} M (1 µM) all-*trans*-retinoic acid (RA) - hereafter referred to as RA medium. RA was prepared as a stock solution at 10^{-2} M in 95% ethanol and stored at -20°C. The stock solution was diluted directly into the culture medium to obtain the desired concentration of 10^{-6} M (Jones-Villeneuve *et al.* 1982). Medium containing RA was renewed every 3-4 days. Once the OP cells had reached a confluency of 60-70% at 33°C, they were shifted to 39°C for the required length of time and processed depending on the experiment. Cells were grown in parallel under standard conditions of DM-10 containing Gentamycin.

2.2.7 *Soft agar assay*

To determine whether the OP cell lines were transformed or immortalised, the cells were grown at 33°C in soft agar (Paul 1975). In soft agar, transformed cells will grow and multiply as a colony while immortalised cells will remain as a single cell in the agar. As a control, the transformed K562

cells (Table 2.1, A gift from Dept. of Immunology, University of Cape Town) (Lozzio & Lozzio 1975) were simultaneously cultured at 37°C.

A 1.32% aqueous solution of Difco Bacto agar was sterilised by autoclaving and maintained at 40°C until ready for use. DMEM was prepared at double strength, 10% FCS added and heated to 37°C. The cells were then suspended at double density, 2x 10^3 cells/ ml in 2 ml 2x DM-10. Equal parts of agar and cell suspension were mixed and 2 ml of this mixture was plated out in 35 mm dishes in duplicate. The agar/cell mixture was allowed to set at RT for 5-10 minutes, then at 4°C for 2-3 minutes. The dishes were incubated at 33°C for the OP cell lines and at 37°C for the K562 cell line for a period of 21 days. The medium was supplemented every 4-5 days with 500 µl of 1x DM-10.

2.3 Immunocytochemistry (ICC)

Immunocytochemistry with specific antibodies was used to characterise the phenotype of the OP cells. Antibodies that were specific to either the olfactory neuroepithelium or to different cell types; neurons, glia or oligodendrocytes were used to characterise the cell lines. Details of the antibodies that I used are summarised in Table 2.2.

2.3.1 *Immunocytochemistry on the OP cell lines*

Cell monolayers that were approximately 70% confluent on Thermonox coverslips were treated according to the standard protocol summarised in Fig 2.1.

Staining with the monoclonal primary antibody (PAb 101, ATCC, Gurney, 1980), directed against the C-terminus of the intracellular SV40 Tag, required that the cells be permeabilised before application of the first antibody. This was achieved by exposing the fixed cells to 100% methanol for 15 minutes at -20°C (immediately after step 'a' in the standard protocol. In this case I used PBS containing 1% FCS for the washes and to dilute the primary antibody. In all other respects the protocol was the same.

2.3.2 *BrdU (5-Bromo- 2'-deoxyuridine) incorporation*

OP cells grown on glass coverslips to semi-confluency (33°C) or for 10 days (39°C) were placed in DM-10 containing 10 μM BrdU for 2 hours (33°C) or for 2 hours or overnight (39°C). The cells were then fixed in 4% PFA, washed (PBS x3), and lysed in 4 M HCl for 20 minutes at RT. After washing in buffer 1 (0.5% BSA, 0.1% Tween 20 in PBS) three times, the cells were incubated with mouse monoclonal antibody (clone BMC 9318, Ig G1) to BrdU for 1 hour at 37°C, washed and treated with anti-mouse Ig-alkaline phosphatase (Ig-AP) for 45 minutes at 37°C. I then rinsed the coverslips in PBS and immersed them in nitroblue tetrazolium (0.33 mg/ml) / 5-bromo-4-chloro-3-indolyl phosphate (0.165 mg/ml) (NBT/BCIP) prepared in substrate buffer [100 mM NaCl; 50 mM $MgCl_2$; 100 mM Tris-HCl pH 9.5]. The cells were then dehydrated, cleared and mounted in Histomount. NBT and BCIP was prepared as a 50 mg/ml stock solution in 70% Dimethylformamide (DMF) or 100% DMF, respectively and stored at 4°C as described.

Cells in culture

↓

[1] Wash

a) Fix (4% paraformaldehyde in PBS 30 minutes, 4°C)

↓

Wash

b) [2] Block (1% H_2O_2 in methanol, 15 minutes, RT)

↓

Wash

c) Block (PBS containing 10% FCS, 0.1% triton X-100; 1 hour; RT)

↓

Wash

d) [3] Incubate with first antibody diluted in PBS containing 5% FCS (Overnight; 4°C)

↓

Wash

e) [4] Incubate with biotinylated secondary antibody diluted in PBS containing 5% FCS (1 hour; RT)

↓

Wash

f) Incubate with streptavidin-peroxidase conjugated 1:1500 in PBS (1 hour; RT)

↓

Wash

g) [5] Incubate with substrate (DAB + H_2O_2)

↓

Wash

h) Counterstain with Hematoxylin (1g/L)

↓

Wash

i) [6] Dehydrate through ethanol series and clear with xylol

↓

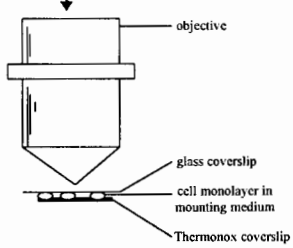
Wash

j) [7] Mount on 0.17 mm thick glass coverslips with mounting medium

↓

k) Examine at 20x and 40x magnification

↓



NOTES

1. Each wash involved rinsing the coverslip for 5 minutes in changes of PBS.

2. For the first blocking step for the O1 antibody I used 1% hydrogen peroxide (H_2O_2) in PBS for 15 minutes at RT.

3. The antibody specificities and the concentration at which I used them are given in Table 2.2.

4. The specificities of the second, biotin-labelled antibodies was appropriate for the species and the class of the first antibody. Polyspecific goat anti-mouse Ig G/M/A (Sigma B2016) was used at an optimal dilution of 1:250. Goat anti-rabbit Ig G heavy and light chain (Vector laboratories) I used at a dilution of 1:250.

5. The peroxidase substrate, 3, 3- diaminobenzidine tetrahydrochloride (DAB 0.6 mg/ml) and H_2O_2 (0.018%) were dissolved in PBS.

6. The coverslips where then taken through the following series of ethanol concentrations for 2 minutes each: 100%; 96% ; 80%, 70%.

7. By mounting the preparation on a glass coverslip I was able to focus on the cellular level with a 20x and a 40x objective. The Thermonox coverslip did not allow one to do this.

Fig 2.1 Protocol for the Immunocytochemistry method

TABLE 2.2 *List of antibodies and their dilutions used in this study*

Primary antibody	Dilution	Source	Secondary antibody	Dilution
Nestin, polyclonal	1:2500	R.D.G McKay, NIH, Bethasda	Biotinylated-conjugated goat α-rabbit Ig G/H/L	1:250
Vimentin, monoclonal (clone V9)	1:10	Boehringer Mannheim	Biotinylated-conjugated goat α-mouse Ig G/M/A	1:250
Neurofilament 160 (NF160), monoclonal (NN18)	1:100	Sigma	Biotinylated-conjugated goat α-mouse Ig G/M/A	1:250
GFAP, monoclonal (GA-5)	1:100	Boehringer Mannheim	Biotinylated-conjugated goat α-mouse Ig G/M/A	1:250
Keratin (Z622)	1:750	Dako	Biotinylated-conjugated goat α-rabbit Ig G/H/L	1:250
Oligodendrocyte 01, monoclonal (clone 59)	1:50	Boehringer Mannheim	Biotinylated-conjugated goat α-mouse Ig G/M/A	1:250
SV40 large T antigen (Pab 101)	1:1	ATCC, Gurney 1980	Biotinylated-conjugated goat α-mouse Ig G/M/A	1:250
BrdU (5-bromo-2`-deoxyuridine), mouse monoclonal	1:10	Boehringer Mannheim	α-mouse Ig-alkaline phosphatase	1:10

2.4 Nucleic Acid Methodology

For the most part I have used techniques that have come to be regarded as standard and are described in detail in such works as Ausubel *et al.* 1997; Sambrook *et al.* 1989. The following brief descriptions summarise the main procedures that I used.

2.4.1 *Isolation of genomic DNA*

I looked for the integration of proviral DNA genomic DNA extracted from the OP cell lines as follows: Cell pellets from cultures grown at 33°C to ~80% confluency were resuspended in lysis buffer (0.6% SDS, 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 50 mM EDTA) and incubated at 37°C for 30 minutes with shaking. Protenaise K was added to a final concentration of 0.4 mg/ml and the suspension was incubated for a further 5 hours at 37°C with slow shaking. The DNA was then purified with phenol, phenol-chloroform and chloroform. Genomic DNA was precipitated in 0.3 M sodium acetate pH 5.2 and 2.5 volumes cold ethanol; washed in 70% ethanol and resuspended in 200-300 µl nuclease-free water.

2.4.1.1 Southern blot analysis

For Southern blot analysis, 20 µg of Bgl II-digested genomic DNA was electrophoresed on a 1% Agarose gel alongside a λ-PstI marker. The DNA fragments were transferred onto a Hybond N+ nylon membrane and fixed with a UV crosslinker. I prehybridised the membranes in rapid hybridisation buffer for one hour at 60°C and then exposed them overnight at 60°C to rapid hybridisation buffer containing a randomly primed, ^{32}P labelled-808 bp Xba1-BglII fragment containing the neomycin resistance gene from pGK-lacZ (Table 2.3) (R. Jaenisch, Massachusetts Institute of Technology, Cambridge). This probe was used to analyse the insertion site of the retrovirus into the mouse genome. The membranes were then washed sequentially at 65°C in 2x SSC twice for 10 minutes; 1x SSC/0.1 % SDS for 15 minutes and finally in 0.2x SSC/0.1 % SDS twice for 10 minutes. The membranes were then sealed in plastic bags and exposed to autoradiographic film (Hyperfilm MP) for 3 days. For random prime ^{32}P labelling, I used commercially available kits (Megaprime kit or Prime-a-gene kit) according to the manufacturer's instructions.

2.4.2 *Isolation of total RNA*

Total RNA was extracted using one of two methods depending on the quantity of material available. For large quantities, the single step guanidinium thiocyanate method (Chomczynski & Sacchi 1987) was used and for small quantities, the TRI reagent method was used (Chomczynski 1993 and Tri reagent 1995).

2.4.2.1 Single step method for total RNA isolation

Cells grown in tissue culture were harvested and pelleted at 1000g for 5 minutes. The cell pellet was resuspended in 500 µl of solution D (Section 2.9). Sequentially, 0.05 ml 2 M sodium acetate pH4, 0.5 ml phenol pH4 (water-saturated) and 0.15 ml chloroform-isoamyul alcohol mixture (49:1) were added to the cell homogenate with thorough mixing. The solution was then cooled on ice for 15 minutes and centrifuged at 15000g for 20 minutes at 4°C. The aqueous phase containing the RNA was transferred to a fresh tube, mixed with 0.5 ml isopropanol and placed on dry ice for 10 minutes to precipitate the RNA. The tube was centrifuged (15000g; 4°C; 20 minutes); the RNA pellet was dissolved in 0.15 ml solution D and re-precipitated by adding 0.15 ml isopropanol and placing the tube on dry ice for 10 minutes. The final RNA pellet obtained after centrifugation (15000g; 20 minutes; 4°C) was washed with 75% ethanol and dissolved in 50 µl nuclease-free water by heating to 65°C.

2.4.2.2 TRI reagent method for isolation of total RNA

A cell pellet containing ± $5x10^6$ cells or less was resuspended in 500 µl of TRI reagent and incubated for 5 minutes at RT. Phase separation was induced by the addition of 0.05 ml 1-Bromo-3-Chloropropane (BCP) and the sample was shaken vigorously for 15 seconds. The homogenate was then incubated for 15 minutes at RT and centrifuged at 12000g for 15 minutes at 4°C. The aqueous phase containing the RNA was transferred to a fresh eppendorf, precipitated with 250 µl of isopropanol at RT for 5-10 minutes, recovered by centrifugation (12000g; 4°C; 20 minutes), washed with 75% ethanol and dissolved in 10-15 µl of nuclease-free water at 65°C.

2.4.3 *Reverse transcription polymerase chain reaction (RT-PCR)*

Total RNA (10μg) was treated with 1U/μl Rnase-free DNase to digest any contaminating DNA present. The solution was purified with phenol-chloroform pH 4 (1:1) and the RNA recovered by precipitation with 0.3 M sodium acetate pH 5.2, 2 volumes of ethanol and 1/100th volume of a stock solution of a commercially obtainable high molecular weight polymer of α-D-glucose, Microcarrier gel. The RNA pellet was washed with 75 % ethanol and resuspended in 12 μl of nuclease free water. The quality of RNA was established by electrophoresis on a 1 % Agarose-MOPS gel.

For first strand synthesis, 5 μg of DNase-treated RNA (denatured at 70°C for 10 minutes, then placed on ice) was used as a template in the following reaction mixture: 200 ng random primers, 1x M-MLV reaction buffer, 0.5 mM dNTP mix, 20 U RNasin and 200 U M-MLV reverse transcriptase enzyme in a total volume of 20 μl. The reaction was allowed to proceed at RT for 10 minutes; 37°C for 50 minutes; 70°C for 15 minutes and stopped on ice. For each 50 μl PCR reaction, 1 μl of the reverse transcription reaction was used. The PCR reactions were optimised for each primer pair (Table 2.4). Negative PCR controls contained no DNA or DNA from a negative cell line, COS-1 (Table 2.1) that does not express olfactory and neuronal markers. Positive controls came from a cDNA clone or from genomic DNA specific for that primer pair.

PCR was performed in an Omnigene Hybaid PCR machine with a heated lid. The basic conditions for the PCR reactions were as follows. For the *β-actin primers*, the following conditions were used: 1x Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.3 μM each of the forward and the reverse primers and 0.1 U Taq Thermoprime DNA polymerase. The PCR cycling was as follows: 95°C for 2 minutes (1 cycle), 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds (30 cycles), 72°C for 5 minutes (1 cycle). The specific conditions by which the PCR reaction for each primer set (Table 2.4) deviated from the standard protocol are listed below.

Olf-1 primers; 0.5 μM of each primer was used with an annealing temperature of 60°C.

Pax6 primers; 4.5 mM $MgCl_2$, was used with the cycling parameters of 95°C for 2 minutes (1 cycle), 94°C for 25 seconds, 52°C for 40 seconds and 72°C for 1 minute (40 cycles), 72°C for 5 minutes (1 cycle).

Nestin primers; 4.5 mM $MgCl_2$ and 5% formamide was used in the PCR reaction, with an annealing temperature of 50°C.

Table 2.4 *List of Primers and their PCR product size*

Name	Sequence	PCR size	Reference
β-actin F[a]108	5'AGCCATGTACGTAGCCATCCAG3'	G[d] = 806 bp C[e] = 365 bp	E.Vreugenhill Personal communication
β-actin R[b]109	5'GGAGTACTTCTAGGACTCGCTCG3'		E.Vreugenhill
β-actin I[c]480	5'AGCCATGTACGTAGCCATCCAG3'		This study
Olf-1 F187	5'TGTCCACAATAACTCCAAGCACGG3'	C = 300 bp	This study
Olf-1 R188	5'CAGAACTGCTTGGACTTGTACGAC3'		This study
Olf-1 Ioligo2	5'CTGAATTCTCCTCATGCCATCCGAGT T3'		This study
Pax-6.43 F	5'CACCAACTCCATCAGTTCTAACGG3'	C = 750 bp	A.Chess; Personal communication
Pax-6.44 R	5'CGATCACATGCTCTCTCCTTCTCTC3'		A.Chess
Pax-6 I	TCACATCAGGTTCCATGTTGG3'		This study
GnRH F800	5'TCTACTGCTGACTGTGTGTTTG3'	G = 2920 bp C = 242 bp	This study
GnRH R801	5'TCTTCTTCTGCCTGGCTTC3'		This study
GnRH I802	5'GCCAGTGGACAGTACATTCGAAG3'		This study
Nestin F150	5'AATCTTTTCAGATGTGGGAG3'	C = 250 bp	Kachinsky et al.1994
Nestin R402	5'GCCACGCTCTCCAGCTCTT3'		Kachinsky et al.1994
Nestin I203	5'GTCAAGACGCTAGAAGAGCA3'		Kachinsky et al.1994
BF-1 Fs10	5'ACGAGGATCCGGGCAAGGGC3'	C = 350 bp	E.Rumbak Personal communication
BF-1 R1474	5'TACGAATTCGGTGGAGAAGGAGTGG 3'		E. Rumbak
Mash-1 F	5'CTCTTAGCCCAGAGGAAC3'	C = 454 bp	Calof et al. 1996
Mash-1 R	5'GGTGAAGGACACTTGCAC3'		Calof et al. 1996
Mash-1 1315	5'GATGCAATGTGCTCAGCAAC3'		This study

Name	Sequence	PCR size	Reference
Otx-2 F	5'CTCTAGTACCTCAGTCCC3'	C = 242 bp	Calof et al. 1996
Otx-2 R	5'GTCCAGGAAGCTGGTGAT3'		Calof et al. 1996
$p75^{NGFR}$ F	5'CAAGGAGACATGTTCCACAGG3'	C = 840 bp	Roskams et al.1996
$p75^{NGFR}$ R	5'CAGAGATGCCACTGTCGCTGT3'		Roskams et al.1996
pGEM-T T7	5'ATTATGCTGAGTGATATCCCGCT3'	695 bp	Promega
pGEM-T SP6	5'TTTAGGTGACACTATAGAATAC3'		Promega
OR F3 [f]	5'AGATCTAGATGGCITAT(C)GAT(C)C(A)GITA(T)T(C)GTIGC3'	C = 550 bp	G.Vassart Personal communication
OR R7 [f]	5'CTTAAGCTTA(G)AAIGGA(G)TTNAGCATNGG3'		G.Vassart
OR RC[f]	5'GCTCTAGATAA(GT)ATA(G)AAIGGA(G)TTIAA(G)CAT3'		Ngai et al. 1993a

[a] = Forward primer
[b] = Reverse primer
[c] = Internal oligonucleotide
[d] = Genomic DNA PCR product size
[e] = cDNA PCR product size
[f] = Restriction enzyme sites at 5' end of primers (Bgl II and Xba I for OR F3, Afl II and Hind III for OR R7 and Xba I for OR RC).

BF-1 primers; 3mM $MgCl_2$, 5% formamide and 0.4 μM of each primer was used with an annealing temperature of 59°C.
Olfactory receptor transcripts primers; 3 mM $MgCl_2$ and 0.5 μM of each primer was used with the cycling parameters of 94°C for 4 minutes, 50°C for 2 minutes and 72°C 3 minutes (1 cycle), 94°C 1 minute, 60°C 2 minutes, 72°C 3 minutes (29 cycles) and 72°C for 10 minutes.
Mash-1 and Otx2 primers; were used exactly as described by Calof *et al.* (1996).
$p75^{NGFR}$ primers; the cycling parameters were 95°C for 3 minutes (1 cycle), 94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute (35 cycles) and 72°C for 5 minutes.

Half of the products of the PCR reaction was electrophoresed on a 2% Agarose/TAE gel along with a 100 bp DNA ladder marker. PCR results were confirmed and sensitivity of detection was increased by transferring the PCR products to Hybond N+ nylon membranes in 10x SSC and probing with a cDNA probe or an internal gene specific oligonucleotide. I refer to this procedure in later sections as internal oligonucleotide hybridisation. The membrane was fixed by UV crosslinking. The membranes were probed with either ECL non-radioactively end- labelled internal oligonucleotide probes or an ECL random-labelled cDNA probe. The detection probes were designed to hybridise with an internal site present in the expected PCR product (Table 2.4). Non-radioactive labelling and subsequent detection were done according to the manufacturer's instructions. The hybridisation temperatures were calculated according to the manufacturer's instructions and they were: 42°C for the Pax6, GnRH and the nestin probes and 50°C for the β-actin and the Olf-1 probes.

2.4.4 *Cloning of RT-PCR products*

The PCR products were excised from a 1% low melting point Sea Plaque agarose gel and purified using the Geneclean II purification kit. The purified PCR product was then ligated into the pGEM-T vector using T4 DNA ligase. The ligation reaction was allowed to proceed overnight at 4°C, after which $1/20^{th}$ of the reaction mixture was used to transform competent *E. coli* DH5α cells (Table 2.3) by electroporation.

To prepare competent cells, a single colony of *E. coli* cells was grown overnight in SOC media to an OD_{600} of ± 0.5. The culture was chilled on ice for 30 minutes and then centrifuged at 4000g for 10 minutes. The pellet was washed 3 times by resuspending and centrifugation (4000g; 4°C; 10 minutes) in ice-cold 10% glycerol and finally resuspended at $5x10^{11}$cells/ml in the same solution. Separate 40 μl volumes of this suspension was stored at -70°C.

To transform the cells, I introduced 40 µl of cell suspension and 1/20th volume of ligation mixture into cuvettes with a 0.2 cm electrode gap. The electroporator was set at: 2.5 kV, 25 µF and 200 Ω to generate a field strength of 12.5 kV/cm and a pulse administered. Immediately thereafter I added 1 ml of SOC medium and incubated the cells with shaking at 37°C for 1 hour before plating onto Luria Bertania broth (LB) plates containing 100 µg/ml ampicillin, 40 µg/ml X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.5 mM IPTG (Isopropyl-β-D-Thiogalactopyranoside) (Nickloff 1995 and Ausubel *et al.* 1997) White colonies were selected the following day for colony PCR or for minipreparations of plasmid DNA.

2.4.5 *Preparation of plasmid DNA and sequencing*

Restriction enzyme analysis of purified plasmid DNA or colony PCR was routinely used to analyse transformants.

For the first method, I prepared alkaline lysis minipreps of plasmid DNA (Birnboim & Doly 1979; Birnboim 1983), digested these with SphI and PstI (which cut within the multiple cloning site of the pGEM-T vector) and analysed the fragments by electrophoresis.

For colony PCR, a single colony was suspended in 20 µl of water, of which 5 µl was used in the final PCR reaction of 20 µl. The PCR reaction contained 0.2 mM dNTP mix (dATP, dCTP, dGTP and dTTP), 1.5 mM $MgCl_2$, 1x Taq buffer [200mM $(NH_4)_2SO_4$, 750mM Tris-HCl pH 9.0 and 0.1% (w/v) Tween], 0.625 pmoles of each primer T7 and SP6 (Table 2.4) and 2.5 U of Taq Thermoprime^{Plus} DNA Polymerase. The cycling parameters for the PCR reactions were as follows: 95°C for 5 minutes (1cycle), 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds (30 cycles) and 72°C for 5 minutes (1 cycle). For confirmation of insert, the entire PCR product was loaded onto a 2% agarose gel.

Colonies which gave positive PCR results were grown in large volumes to prepare substantial amounts of pure plasmid DNA using a commercially available column protocol (Wizard Midi prep kit -Promega). Purified plasmid DNA (5 µg) was sequenced with the T7 and SP6 primers (Table 2.4) on an automated DNA sequencer (ALFexpress Automatic DNA Sequencer, Pharmacia Biotech AB S-751 82 Uppsala, Sweden). The DNA sequences were compiled using the GCG DNA analysis software program (Wisconsin Package Version 9.1, Genetic Computer Group (GCG), Madison, Wisconsin). Comparative searches at the nucleotide and at the protein levels were performed against

the Genbank, EMBL (European Molecular Biology Laboratory), the Swiss-Prot databases and the olfactory receptor database (public-http://senselab.med.yale.edu/ORDB/).

2.5 Light microscopy

The OP cell lines were checked each day using a Nikon inverted microscope fitted with a 20x objective and green filter. For morphological studies the cells were grown in 100 mm TC dishes. The cells were viewed and photographed directly in these dishes on an inverted Nikon microscope at 20x magnification. The magnification to the camera was 2.5x. For Immunocytochemistry, the cells were grown on Thermonox round coverslips. These coverslips allow the cells to grow well and remain fixed throughout the process of ICC. However the Thermonox was not suitable for high power microscopy. To overcome this, the Thermonox coverslips were mounted on 0.17 mm thick rectangular glass coverslips. This allowed me to view the cells through the glass at 20x and 40x magnification. For black and white photography, Kodak technical pan was used and for colour photography, either Agfa or Konica, at ASA 100 was used.

2.6 Genbank accession numbers

The following sequences were submitted to genbank: OR6a (AF042359), OR27a (AF042360) and OR55a (AF042361).

Table 2.1 *Table of cell lines used and constructed*

Cell line	Description	Reference /Source
OP6	Derived from mouse E10.5 olfactory neuroepithelium	This study
OP27	Derived from mouse E10.5 olfactory neuroepithelium	This study
OP47	Derived from mouse E10.5 olfactory neuroepithelium	This study
OP55	Derived from mouse E10.5 olfactory neuroepithelium	This study
OP4-18	Derived from mouse E10.5 olfactory neuroepithelium	This study
OP5-1	Derived from mouse E10.5 olfactory neuroepithelium	This study
GT1-7	Immortalised hypothalamic cell line that expresses a neuronal phenotype and contains GnRH and GAP immunoreactivity	Mellon et al. 1990 Prof. J. Roberts (Fishberg Center for Neurobiology, Mount Sinai School of Medicine, New York)
3T3	Spontaneously immortalised mouse fibroblast cell line	Prof. J. Roberts (Fishberg Center for Neurobiology, Mount Sinai School of Medicine, New York)
COS-1	African monkey kidney cells transformed with the SV40 T-antigen	Gluzman, Y., 1981. A gift from the Department of Chemical Pathology, University of Cape Town
ψ2 cell line	Cell line transfected with the plasmid, pZipNeo-SV40U19tsA58	A gift from R. McKay (Almazan & McKay 1992)

Table 2.3 *List of plasmids and the bacterial strain used or constructed in this study*

Plasmids	
pGEM-T	TA cloning vector for PCR products (Promega) - Origin of replication, f1; T7 and SP6 RNA polymerase promoters; multiple cloning sites with α-peptide coding region of the β-galactosidase
pGK-lacZ	Plasmid containing the neomycin resistance gene which was excised with XbaI and BglII (R.Jaenisch, Massachusetts Institute of Technology, Cambridge)
OR6a	Taste receptor clone from OP6 cell line cloned in pGEM-T
OR6b	Taste receptor clone from OP6 cell line cloned in pGEM-T
OR27a	Olfactory receptor clone from OP27 cell line cloned in pGEM-T
OR27b	Olfactory receptor clone from OP27 cell line cloned in pGEM-T
OR47a	Taste receptor clone from OP47 cell line cloned in pGEM-T
OR47b	Taste receptor clone from OP47 cell line cloned in pGEM-T
OR55a	Olfactory receptor clone from OP55 cell line cloned in pGEM-T
OR55b	Clusterin clone from OP55 cell line cloned in pGEM-T
BF-1 cDNA probe	SmaI digest to yield 565 bp fragment corresponding to the PCR product generated with the above primers (E. Rumbak; personal communication)
Bacterial stain	
DH5α	F^- endA1 hsdR17(r_k^-, m_k^+) supE44 λ^-thi-1 recA1 gyrA96 relA1 Φ80dlacZΔM15Δ(lacZYA-argF)U169

2.7 Materials and Suppliers

Table 2.5 *List if materials used in this thesis and their suppliers*

Materials	Suppliers
Autoradiographic film (Hyperfilm MP)	Amersham
Bacto Agar	Difco
BCP (1-Bromo-3-Chloropropane)	Molecular Research Centre, Inc., Cincinatti
BCIP (5-bromo-4-chloro-3-ondolyl phosphate)	Boehringer Mannheim
Bgl II restriction enzyme	Amersham
Biotin-conjugated goat α-rabbit Ig G/H/L	Vector Laboratories Inc.
BrdU (5-bromo-2'-deoxyuridine)	Boehringer Mannheim
BSA Fraction V	Sigma
CMF-HBSS (calcium- and magnesium- free Hanks balanced salt solution)	Gibco BRL
Choroform	Sigma
DAB (3,3-diaminobenzidine tetrahydrochloride)	Sigma
DMEM (Dulbecco's modified Eagle's medium), high glucose	Gibco BRL
DMSO (Dimethyl sulphoxide)	Riedel-de Haen
DNA Ladder marker (100bp)	Promega
DNA sequencer (Automated)	ALF Express, Pharmacia/LKB)
dNTP mix	Promega
ECL 3' oligo labelling and detection system	Amersham
ECL random labelling and detection system	Amersham
EDTA (Ethylene diamine tetracetic acid)	Sigma
Entellen	Merck
Ethanol	Merck
F12 medium	Sigma
Fetal calf serum	Delta Bioproducts
Geneclean II purification kit	Bio 101
Genetecin (G418)	Sigma
Gentamycin	Sigma
Glass coverslips (0.17 mm thick nr.1)	Marienfeld
glycerol	BDH Limited
Guanidinium thiocyanate	Merck
Hanks balanced salt solution (HBSS)	Gibco BRL
Hematoxylin	Merck
HCl	NT laboratories suppliers
Histomount	Zymed
Hoescht stain	Sigma
Hybond N+ nylon membrane	Amersham
Hybridisation Oven	Amersham
Hydrogen peroxide (H_2O_2)	Univ AR
Insulin	Sigma
IPTG (Isopropyl-β-D-Thiogalactopyranoside)	Promega
Isoamyl alcohol	Sigma
Isopropanol	Sigma
Megaprime labelling kit	Amersham

Materials	Suppliers
Methanol	BDH Laboratories supplies
Microcarrier	Molecular Research Centre Inc., Cincinatti
MOPS (3-[N-Morpholino] propane-sulfonic acid)	Sigma
Nitroblue tetrazolium (NBT)	Boehringer Mannheim
Paraformaldehyde (PFA)	Sigma
Penicillin	Sigma
pGEM-T vector	Promega
Phenol (water-saturated, pH 4)	Sigma
Polybrene (Hexadimethrine bromide)	Sigma
Polyornithine	Sigma
Prime-a-gene kit	Promega
Progesterone	Sigma
Pst I restriction enzyme	Promega
Putrescein	Sigma
Random primers	Promega
Rapid hybridisation buffer	Amersham
Retinoic acid (all-trans)	Sigma
Reverse Transcriptase enzyme	Promega
Rnase-free DNase (RQ1 1U/ µl)	Promega
RNasin	Promega
Sea Plaque low meltingpoint agarose	FMC Bioproducts
Sodium Bicarbonate ($NaHCO_3$)	Sigma
SDS (Sodium dodecyl sulphate)	Sigma
Sodium Selenite	Sigma
Sph I restriction enzyme	Promega
Streptavidin-POD	Boehringer Mannheim
Streptomycin	Sigma
T4 DNA ligase	Promega
Thermonox coverslips	Nalge Nunc International
Thermoprime ^{Plus} DNA polymerase	Southern Cross Biotechnology
Tissue culture flasks and plates	Nalge Nunc International
Transferrin	Sigma
Tri Reagent	Molecular Research Centre, Inc.,
Triton X-100	Unitek
Trypan blue	Sigma
Trypsin	USP grade Gibco BRL
Tween 20	Biorad Laboratories
UV crosslinker	Amersham
water, nuclease-free	Sigma
X-gal (5-Bromo-4chloro-3-indolyl-β-D-galactopyranosideside)	Boehringer Mannheim
Xylol	Univ AR Laboratories
Wizard Mini Prep kit	Promega

2.8 Media and Solutions

Alkaline phosphatase substrate buffer:

100 mM NaCl

50 mM $MgCl_2$

100 mM Tris-HCl pH 9.5

Alkaline phosphatase substrate:

0.33 mg/ml NBT (50 mg/ml stock in 70% DMF)

0.34 mg/ml BCIP (50 mg/ml stock in 100% DMF)

0.1 mM levamisol (1 M stock)

Alkaline phosphate substrate buffer

DAB substrate:

0.6 mg/ml DAB (40 mg/ml stock)

0.018 % hydrogen peroxide

1X PBS

Defined N2 medium:

1X DMEM

1X F-12 medium

5 μg/ml insulin (4 mg/ml stock)

100 μg/ml transferrin (10 mg/ml stock)

100μM putrescein (1 M stock in DMEM)

10 nM progesterone (200 μM stock in DMEM, first dissolved in absolute ethanol)

30 nM sodium selenite (300 μM stock in DMEM)

Defined N2 medium containing retinoic acid:

Defined N2 medium

10^{-6} M retinoic acid (0.1 M stock in 95 % ethanol)

50 μg/ml gentamycin (50 mg/ml)

2% FCS

DM-10 TC medium:

1X DMEM, high glucose

3.7 g/ml $NaHCO_3$

Filter sterilised

10% FCS, heat inactivated (58°C 30 minutes)

Genomic DNA lysis buffer:

0.6% SDS (10% Stock)

20 mM Tris-HCl pH 7.5 (1 M stock)

100 mM NaCl (5 M stock)

50 mM EDTA (0.5 M stock)

1X MOPS - RNA running buffer pH 7.0:

20 mM MOPS pH 7.0

5 mM sodium acetate

1 mM EDTA

Phosphate buffered saline (PBS) pH 7.5:

140 mM NaCl

2.7 mM KCl

8 mM $Na_2HPO_4.7H_2O$

1.4 mM KH_2PO_4

0.7 mM $CaCl_2$

0.8 mM $MgCl_2$

SOC medium for E. coli:

2% tryptone

0.5% yeast extract

10 mM NaCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM glucose

Solution D for RNA extraction:

4 M guanidinium thiocyanate

25 mM sodium citrate pH 7 (1 M stock)

0.5% sarcosyl (10 % stock)

0.1 M 2-mercaptoethanol

1X TAE - Tris Acetate buffer:

0.04 M Tris acetate

0.001 M EDTA pH 8.0

Glacial acetic acid

Trypsin/EDTA solution:

0.25% Trypsin

0.02% EDTA (2% Stock)

1X PBS

Chapter 3

Immortalisation and characterisation of olfactory placode cell lines with the temperature sensitive SV40 retrovirus.

3.1 Summary

I have established permanent cell lines by infecting olfactory neuroepithelium primary cultures with a retrovirus carrying a temperature sensitive alleles of SV40 Tag (tsA58, U19). Infected cells were selected with the antibiotic G418. Sixty cell lines were isolated of which 48 survived without crisis or contamination. These 48 cell lines were grown at the permissive temperature of 33°C, total RNA was isolated and analysed by RT-PCR using primers designed to detect transcripts of olfactory or neuronal markers. These markers included Olf-1, BF-1, Pax6, Nestin, GnRH, Olfactory receptor transcripts, Mash-1 and $p75^{NGFR}$. Primers designed to amplify β-actin transcripts were used as an internal control. Four cell lines: OP6, OP27, OP47 and OP55, which expressed different combinations of these markers, were selected for further study.

At the permissive temperature of 33°C, the four cell lines differed morphologically and they divided with different doubling times. Although immortal in the sense that the cultures could be passaged without senescence for 2 years, the cells did not show anchorage independence in soft agar. The OP cell lines all expressed SV40 Tag. At the non-permissive temperature of 39°C, SV40 Tag expression was markedly decreased and proliferation slowed. 5-Bromo-2`-deoxy uridine (BrdU) incorporation studies showed that the cells did not divide at 39°C. Southern blot analysis showed that the four cell lines I developed were clonal. These cells are thermolabile inasmuch as they proliferate at 33°C but cease dividing at 39°C.

3.2 Introduction

3.2.1 *Why immortalise cell lines using the thermolabile SV40 Tag?*

The *ex vivo* culture of living cells or tissues originated in the late nineteenth century in the hope that, by reducing living systems to their essential isolated components, the complexities of *in vivo* organisation would be unravelled. In many respects this reductionist approach has been justified and many of the hopes have been realised.

Initial success with the cultures of neoplastic tissues soon led to the realisation that malignant cells have a potentially infinite lifespan *in vitro* whereas normal tissues do not. Research into the molecular and cellular reasons for this fundamental difference, aided by parallel technical and conceptual advances in other areas of biology, has brought us to the point where compromise between neoplastic/immortal and normal/short lived has been achieved: it is now possible to develop immortal cell lines that retain, either constitutively or subject to induction, features of the normal, differentiated phenotype. Culture systems such as these have provided many clues to processes involved in cell growth, they have helped to define the ways in which cells interact with each other and they have identified factors required for growth and maintenance of cells in culture. Importantly, and particularly where culture conditions can be manipulated to achieve expression of characteristics associated with the "normal" or "mature" phenotype, they provided some understanding of the mechanisms involved in embryonic development and in the way that primitive cells differentiate, in the mature animal, to ensure the healing of wounds, the replenishment of effeted cell populations and the remodelling of tissues as physiological needs alter. Examples of cell lines of this type are as follows:

The rat PC12 pheochromocytoma cell line is a widely employed cell line for the study of the way in which growth factors control neuronal growth and differentiation (Greene & Tischler 1976; 1982). PC12 cells exposed to nerve growth factor (NGF) cease to proliferate and develop properties characteristic of sympathetic neurons. The cells extend neurites which become electrically excitable and are able to form synapses with muscle cells in culture.

A murine embryonic carcinoma (EC) cell line, P19, yields neurons, astrocytes, oligodendrocytes and microglia in response to retinoic acid. These cells provide a system in which the entire differentiation process, from a very undifferentiated, pluripotent cell to a terminally differentiated neuron can be followed (reviewed by Lendahl & McKay 1990 and Wittemore & Snyder 1996).

Although tumour-derived cell lines such as these have been useful in the hands of neurobiologists and developmental biologists, many of them are of ill-defined origin, they do not constitute a developmental spectrum of cells from a defined stage or location and they have lost many of the properties and developmental programs of the region from which they originated (Whittemore & Snyder 1996). The degree to which they resemble differentiated neurons is highly variable and, typically, they are tumorigenic so precluding their use in transplantation studies.

While, with explant or primary cultures, the origin of the cells is known, the yield of a particular type within such cultures is low, the populations of cells are heterogeneous and the cells only survive for a short period (1-2 weeks or less). Nevertheless, these cultures have proven useful and are still being used today. A number of olfactory cultures which have contributed to the study of olfactory development have been discussed in Chapter 1.

As indicated earlier, the immortalisation of cells by oncogene transduction provides an alternate approach that has the advantage that it yields clonal cell types that can then be fully characterised and maintained indefinitely either in culture or in the frozen state, in liquid nitrogen, for long term studies.

A limitation of this approach stems from the fact that oncogene transduction usually blocks the immortalised cells at a particular stage of differentiation. Furthermore, the introduction of immortalising genes into cells may, when the genes are expressed, disturb the normal cellular physiology.

These problems can, theoretically, be overcome by the use of conditionally immortalising genes. I have used an immortalising system in which a temperature sensitive allele of the SV40 oncogene was integrated into the cellular genome by a replication defective recombinant retrovirus. The oncogene carried two mutations:

- The temperature-sensitive, tsA58 mutant is inactive at the non-permissive temperature of 39°C, and thus frees the cells from the immortalising effect of the SV40 Tag. The non-permissive temperature of this SV40 mutant is also the core body temperature of rodents and this feature would allow transplantation of the cell lines back into a rodent host without the risk of tumour formation.
- The second mutation in this retrovirus, the U19 mutation, has been shown to be more efficient than the wild type SV40 retrovirus in immortalising primary cultures (Almazan & McKay 1992; Jat & Sharp 1986).

3.2.2 *Markers used to identify olfactory neuronal cell lines*

In order to characterise the cell lines, I checked whether several mRNA transcripts for proteins known to be associated developmentally or with the mature olfactory neuronal phenotype are expressed in the cell lines. The following is a list of the markers I sought to detect:

3.2.2.1 Olf-1: A marker of immature and mature neurons in the olfactory epithelium

Olfactory factor-1 (Olf-1) was cloned (Wang & Reed 1993) by selection for transcription factors that bind to a consensus motif YTCCCYRGGGAR, found in the promoters of genes that are expressed in the olfactory neuroepithelium (Kudrycki *et al.* 1993; Wang *et al.* 1993). It was suggested that Olf-1 plays a critical role in regulating genes that function in odorant detection and neuronal turnover (Margolis *et al.*1993).

Initially it was believed that Olf-1 was expressed exclusively by olfactory receptor neurons and their precursors (Wang & Reed 1993). Working independently, however, Hagman *et al.* (1993) isolated the gene for early B-cell factor (EBF) that regulates mb-1 gene expression in pre-B cells. EBF has virtually identical homology to Olf-1 except for eight amino acids introduced by alternative splicing. In the adult, EBF is expressed at high levels in lymphoid, adipose tissues and at low levels in the heart, brain, skeletal muscle and kidney cells (Milatovich *et al.*1994). Olf/EBF is now referred to as the Olf/EBF-like (O/E) transcription factor (Wang *et al.* 1997).

Olf-1/EBF (O/E-1) contains a repeated Helix Loop Helix (rHLH) domain. The helices in this motif are both similar to the second helix in the well characterised basic HLH (bHLH) motif. The rHLH motif lacks the characteristic basic residues found upstream of the bHLH motif (Wang & Reed 1993; Wang *et al.*1997). The rHLH contains α-helical repeats which are important for dimerisation. A DNA binding region has been delineated in the N-terminus of the O/E-1 gene. O/E-1 contains a novel zinc co-ordination motif ($H\text{-}X_3\text{-}C\text{-}X_2\text{-}C\text{-}X_5\text{-}C$) which is important for DNA recognition (Hagman *et al.* 1993, 1995; Kudrycki *et al.* 1993; Wang & Reed 1993; Wang *et al.* 1997).

Expression of O/E-1 in the olfactory system, is first detected at E11 in the olfactory epithelium of the embryo and continues throughout gestation into the mature animal where it is found in the immature, the mature olfactory receptor neurons and some basal cells but not in sustentacular cells

(Davis & Reed 1996; Wang & Reed 1993). O/E-1 expression is also detected in the VNO system (Davis and Reed 1996; Shipley & Smith 1995). O/E-1 is expressed at the time when neuronal precursors exit the cell cycle and start to differentiate, after the formation of the olfactory placode and invagination of the olfactory pit. Davis & Reed (1996) thus proposed that Olf-1 plays a role in olfactory neuronal differentiation during embryogenesis as well as in adult neurons. In the adult olfactory epithelium, Olf-1 is expressed in postmitotic neurons and activates expression of olfactory neuron-specific genes (Kudrycki *et al.*1993; Wang *et al.*1993).

When the O/E-1 gene was disrupted by homologous recombination, the homozygous mutant mice had defects in B-cell development but the olfactory epithelium appeared normal and expressed the olfactory neuron specific genes, olfactory marker protein (OMP) and G_{olf} (Lin & Grosschedl 1995). This contradicted the hypothesis that O/E-1 played a critical role in activation of olfactory specific genes. The discovery of two new members of the O/E family of transcription factors (O/E-2 and O/E-3) by Wang *et al.* (1997) may explain this observation. In the adult, the expression of O/E-2 and O/E-3 is identical to that of O/E-1 and is concentrated in mature olfactory receptor neurons and basal cells. O/E-2 and O/E-3, unlike O/E-1, are not expressed in the spleen. In the mouse embryo, the O/E genes are widely expressed in the developing nervous system and are enriched in a number of structures that mediate sensory activities. This suggests that O/E proteins acting together regulate olfactory gene expression (Wang *et al.*1997). This recent evidence contradicts the assumption at the start of this study, that expression of the Olf-1 in these cell lines could be used as a specific characteristic of immature and mature olfactory receptor neurons. Expression of O/E-1 is widespread in the developing nervous system and is a more general marker of immature neurons.

3.2.2.2 Mash-1; an early precursor marker involved in the development of the olfactory receptor neurons

Mash-1(mammalian homologue of *achaete-scute*-1) was originally identified in a screen for mammalian homologues of the *Drosophila* proneural genes of the *Achaete-Schute Complex (AS-C)*. Guillemot & Joyner (1993) isolated Mash-1 using degenerate primers to *Drosophila AS-C* and the rat *Mash* genes (Johnson *et al.* 1990). Mash-1 is typical of this group of transcription factors which are characterised by having a bHLH domain. Mash-1 expression was shown to be restricted to the CNS from as early as E8.5 and its expression changed with the development of the embryo to an adult mouse. Mash-1 expression in the olfactory epithelium was detected at E9.5 in the mouse embryo (Guillemot & Joyner 1993) before olfactory receptor neuron differentiation (Cuschieri &

Bannister 1975, Graziadai & Monti Graziadei 1979) and could thus play a role in development of olfactory receptor neurons.

The importance of the role that Mash-1 plays in the development of the olfactory neuroepithelium was highlighted in the phenotype of mice carrying a targeted deletion of the Mash-1 gene. There was a reduction in the numbers of several types of neurons in these mice, including the autonomic, enteric and olfactory sensory neurons. Homozygous Mash-1 "knock-out" mice died one day after birth with breathing and feeding defects. Their olfactory epithelia were severely affected and was missing most of the olfactory receptor neurons, horizontal basal cells and globose basal cells. Interestingly, the sustentacular cells were intact (Guillemot *et al.* 1993). This result suggested that Mash-1 plays an important role in the development of olfactory receptor neuron from their precursors. Furthermore, this provided indirect evidence that the horizontal basal cells or a subpopulation of these cells were involved in neurogenesis of olfactory receptor neurons, together with the globose basal cells that are the direct precursors for olfactory receptor neurons.

Expression of Mash-1 in the olfactory neuronal lineage has been examined in primary cultures of embryonic mouse olfactory neuroepithelium and in the adult mice in which olfactory neurogenesis had been stimulated by the removal of the olfactory bulb. These studies suggested that cells that express Mash-1 are the precursors of the INPs which give rise to olfactory receptor neurons (Calof *et al.* 1996; Gordon *et al.* 1995).

In the CNS, a number of possible roles has been assigned to Mash-1. Mash-1 may play an important role in early neuronal development. It could be involved in proliferation, survival or in the initiation of differentiation of neural precursor cells (Guillemot & Joyner 1993). Mash-1 does not commit multipotential cells to a neural fate but rather promotes determination of an already committed neuronal precursor cell (Bang & Goulding 1996; Simpson 1995; Sommer *et al.*1995).

In the olfactory neuronal lineage, Mash-1 is required for the production of new neurons rather than the development of a specific neuronal phenotype (Guillemot *et al.* 1993). It is a marker of neural progenitor cells that give rise to the olfactory receptor neurons. It is expressed prior to neuronal differentiation and activates the bHLH transcription factor, *NeuroD* through a cascade of transcription factors that would then induce neuronal differentiation from Mash-1 positive precursor cells to postmitotic olfactory receptor neurons (Cau *et al.* 1997).

3.2.2.3 Pax6: a marker for supporting cells in the olfactory neuroepithelium

The PAX genes, which were originally identified in *Drosophila*, are a family of developmental control genes that encode transcription factors containing a DNA-binding paired domain and a homeodomain (Gruss & Walther, 1992). Pax6 is a member of the vertebrate PAX gene family and has been implicated in eye and nasal development (Del Rio-Tsonis *et al.* 1995; Gruss and Walther 1992).

Naturally occurring Pax mutations found in humans and mice have shown us the importance of *Pax6* during development (Gruss and Walther 1992). Mutations that reduce *Pax6* dosage caused dominantly inherited eye and nose malformations in man (Aniridia) and mice (*Small eye*) (Hanson & Heyningen, 1995). Since, the severity of the mutate phenotype depends upon whether it is present in heterozygous or homozygous dosage, *Pax6* gene products are thought to be required in distinct concentrations. The activity provided by only one allele is below critical threshold levels, leading to phenotypic alteration - a condition known as semi-dominance (Gruss & Walther, 1992 and Hanson & Heyningen, 1995).

Pax6 is expressed before neural differentiation begins in the primordial cells involved in the generation of the dorsal ventral mosaic organisation of the ventral tube. In the olfactory epithelium, Pax6 is expressed from the relatively primitive olfactory pit to the late nasal structure (Gruss & Walther 1992). Initially, at E8 in the mouse embryo, Pax6 expression is localised in a broad region of the head surface ectoderm, very similar to the expression of Olf-1 (Davis & Reed 1996). As development progresses, the expression pattern becomes restricted to the developing lens and nasal placodes. Pax6 expression is most abundant in the E10.5 embryo. After that, its expression is down-regulated when cells became postmitotic and start to differentiate (MacDonald & Wilson 1996 and Walther & Gruss 1991). The widespread expression of Pax6 in the anterior neural plate and placode-forming epithelium is consistent with a gene defining a field of cells that are competent to form eye and nose tissue.

Later in embryonic development (from E16 embryos) Pax6 immunoreactivity becomes restricted to the horizontal basal cells, the Bowman`s glands and the sustentacular cells – in a pattern which is retained in the adult mouse (Davis & Reed 1996). The localisation of Pax6 to sustentacular cells, horizontal basal cells and Bowman`s glands may reflect a relationship between these cells and provide indirect evidence that the horizontal basal cells and/or the Bowman`s glands are progenitors of the sustentacular cell population. It is, thus proposed that Pax-6 plays different roles early and

late in olfactory development. Early in development, Pax6 is important for neurogenesis, proliferation and in the transition from ectoderm to nasal placode formation while in the later stages of development, it was important in non-neuronal lineage formation (Davis & Reed 1996).

Alternate splicing of the Pax6 gene, where an additional exon (5a) is retained, results in a protein with a 14 amino acid insertion in the paired domain (Epstein *et al.* 1994). Both spliced products are expressed in the developing eye, brain, spinal cord and the olfactory epithelium (Davis & Reed 1996; Ton *et al.* 1991). The two Pax6 paired domains are postulated to function differentially during embryogenesis. A mutation at the alternate splice acceptor site, that changes the ratio of the two isoforms, causes a distinct human ocular syndrome, known as the Bh mutation. The existence of the Bh mutation suggests that the two isoforms are not functionally interchangeable, that the two proteins most likely to regulate different target genes and that they play distinct functional roles in development (Epstein *et al.* 1994).

We have used primers designed to amplify a transcript of Pax-6 that would recognise both forms of Pax-6 in the olfactory system (Table 2.4).

3.2.2.4 BF-1: a transcription factor important for proliferation of progenitor cells

Brain factor 1 (BF-1) was cloned (Tao & Lai 1992) from an embryonic brain cDNA library, by homologous screening for the "winged-helix" family of transcription factors. The name "winged-helix" is derived from the structure of the DNA binding domain of a member of this family, hepatocyte nuclear factor-3 (HNF-3). X-ray diffraction analysis of the DNA binding domain of HNF-3γ/DNA co-crystallised with its DNA response element showed a 3-D structure resembling the shape of a butterfly: a core derived from the α-helical and β-sheet elements flanked by two "wings" W1 and W2 (Clark *et al.* 1993). This family of proteins is also referred to as the forkhead/HNF-3 family of transcription factors, since forkhead in *Drosophila* (Weigel *et al.* 1989) and HNF-3 in rat (Lai *et al.* 1990) were the first members to be identified.

BF-1 expression is first detected at E8.25 in the telencephalon and the ventricular zone of the invaginating tip of the nasal neuroepithelium destined to become the olfactory epithelium. BF-1 expression has been detected in the VNO but not in the optic stalk (Monaghan *et al.* 1995; Xuan *et al.* 1995). In the brain, BF-1 expression is highly restricted to the rostral end of the developing neural tube that gives rise to the region of the brain that subdivides into the forebrain. This

subdivision of the developing brain occurs prior to the differentiation of the progenitor cells into neurons and glia. BF-1 expression peaks between E14-E17 in the embryo, which correlates with the major period of neuronal proliferation. BF-1 expression declines once progenitor cells start to differentiate (Tao & Lai 1992).

Further evidence for a role for BF-1 in growth control comes from BF-1 knockouts. Whereas the heterozygous mutants developed normally, the homozygous mutants die at birth and showed strongly reduced cerebral hemispheres. In new-born BF-1 (-/-) mutants the olfactory bulbs aremarkedly reduced in size and the nasal structures arising from the nasal placodes fail to grow normally (Xuan *et al.* 1995).

It is thought that in the absence of BF-1, neuroepithelial precursor cells withdraw from the cell cycle resulting in the premature onset of neuronal differentiation. BF-1 thus plays an important role in proliferation and controlling the timing and the number of progenitor cell divisions.

3.2.2.5 GnRH neurons: hypothalamic neurons with an olfactory origin

Gonadotropin-releasing hormone (GnRH) expressing neurons have been shown to differentiate from the olfactory placode in the mammalian embryo (Schwanzel-Fukuda & Pfaff 1989; Wray *et al.* 1989 a,b). *In situ hybridisation* histochemistry, immunocytochemistry and [^{3}H] thymidine-birthdating data suggest the following sequence of events during ontogeny of the GnRH neuronal population. GnRH progenitor cells located in the olfactory placode become postmitotic from E10-E11 to produce the total population of GnRH cells. From E12.5 to E15.5, GnRH$^+$ cells migrate through the nasal septum and into the forebrain via the vomeronasal - nervus terminalis nerves. The nervus terminalis is a structure that originates, along with the vomeronasal complex, in the olfactory placode and migrates centrally to contact the developing forebrain (Wray *et al.* 1989b). By E16.5 the adult-like distribution of the GnRH neurons is complete (Schwanzel-Fukuda & Pfaff 1989; Wray *et al.* 1989a).

The use of GnRH expressing cell lines (Radovick *et al.* 1991) provides evidence that there are two spliced variants of proGnRH expressed in the olfactory area (Chapter 1). The primers used in this study to identify the presence of GnRH transcripts were designed across exon 2 and exon 4 (Fig 3.5) to identify the full length mature proGnRH transcript, which is the predominant form in the olfactory system.

3.2.2.6 Nestin: a marker for neural precursor cells

Nestin is an intermediate filament protein that belongs to Class 6 intermediate filaments (Lendahl *et al.* 1990; Chapter 4) and is expressed in proliferating CNS stem cells from as early as E7.75 in the embryo (Dahlstrand *et al.* 1995). The transition from proliferating cells to postmitotic cells results in a rapid decrease in nestin expression. Dahlstrand *et al.* (1995) was unable to detect nestin expression in proliferating cells in the olfactory epithelium using *in situ hybridisation* with oligonucleotides specific to mouse nestin. Consistent with this data, Pixley (1996) was unable to detect nestin protein expression in olfactory neurons or their progenitor cells, when using an olfactory cell culture from postnatal rats. However, nestin antibody strongly stained the Schwann cell-like glial cells. Osada *et al.* (1995) used antibodies to nestin and showed extensive expression within the chemosensory precursor cells of the VNO and proposed nestin as a marker for chemosensory precursor cells. Initially nestin was widely expressed but later its expression became restricted to the basal layer.

3.2.2.7 p75: Low affinity nerve growth factor receptor ($p75^{NGFR}$), a non-neuronal olfactory marker

Within the olfactory epithelium the family of neurotrophin-receptor tyrosine kinases - Trks A, B and C - are expressed sequentially during olfactory neurogenesis (Roskams *et al.* 1996). These receptors bind to different neurotrophins required for the survival and differentiation of specific neural populations during ontogeny. An additional receptor, the low affinity nerve growth factor receptor, p75 ($p75^{NGFR}$) has been cloned and shown to bind to the same neurotrophins as the Trks A, B and C but with a lower affinity (Johnson *et al.* 1986; Radeke *et al.* 1987). Initially this receptor was called low affinity NGFR, because it was identified as a low affinity receptor for NGF but was later also referred to as $p75^{NTR}$ (Carter & Lewin 1997) as it can bind all of the neurotrophins.

Trks A, B and C are expressed in different neuronal subpopulations throughout the olfactory epithelium. Trk A is expressed in the precursor neuronal basal cells at the base of the neuroepithelium, Trk B is detected within the cell bodies and dendrites of immature neurons that have exited the cell cycle and divided into daughter neuroblasts and Trk C is detected within members of mature olfactory receptor neurons (Roskams *et al.* 1996). In contrast to these neuronal expressions of the Trks, $p75^{NGFR}$ is expressed in olfactory ensheathing cells (Gong & Shipley 1996; Li *et al.* 1997) and in non-neuronal glial cells in the lamina propria. It has been proposed that these

non-neuronal cells, situated below the basal cells, sequester NGF and control the supply of NGF to Trk A^+/ NGF-sensitive basal cells, which require NGF for mitosis. $p75^{NGFR}$ has been detected in the cell bodies of neurons that appeared to be degenerating (Roskams *et al.*1996). In the autonomic nervous system, $p75^{NGFR}$ has been used as a marker of undifferentiated stem cells that also express the multipotential stem cell marker, nestin (Sommer *et al.*1995).

In this chapter I describe the immortalisation of cells from primary cultures of E10.5 mouse olfactory placodes and the use of various markers to identify the phenotype of the cell lines. Furthermore, I describe the characterisation of four of the cell lines at 33°C and at 39°C in DM-10 in terms of their growth characteristics, their expression of SV40 Tag protein and their clonality.

3.3 Results

3.3.1 *Infection of olfactory primary cultures with temperature sensitive retrovirus*

The olfactory placodes from E10.5 mouse embryos (Fig 3.1) were removed and plated as partially dissociated explants as described in the methods section (2.3.1). Primary cultures were infected with SV40tsA58/U19 recombinant retrovirus and exposed to selection medium containing G418.

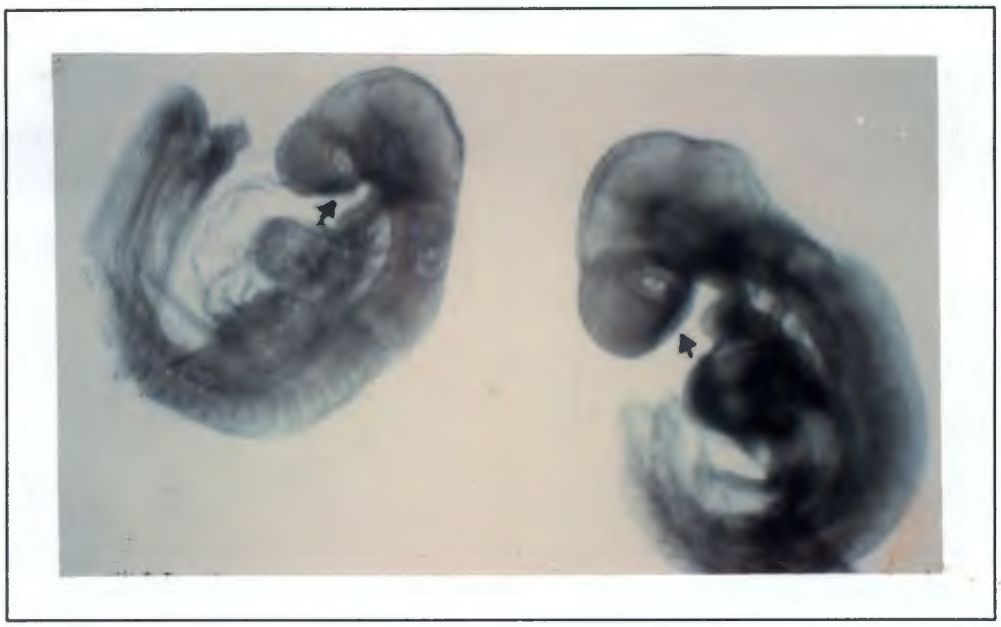


Fig 3.1 Embryonic day 10.5 mouse embryos removed from the placenta of mice and used to isolate olfactory placodes (indicated by the arrows). The olfactory placodes were dissociated for the preparation of primary cultures which were immortalised with the temperature sensitive SV40 retroviral vector.

Sixty different colonies (designated OP6-OP66) were isolated and cloned. Of these, 48 were established as cell lines that survived expansion, sequentially through 96-well, 24-well and 6-well plates and finally into 50 ml flasks. At this stage, test for mycoplasma infection were negative. All of the lines survived freezing, liquid nitrogen storage and thawing without difficulty. They were maintained in DM-10 at 33°C in a humid atmosphere of 10% CO_2 in air without antibiotics.

Although the 48 cell lines were similar at 33°C, they could be divided into those that grew rapidly and those that grew slowly. In addition to the 48 OP cell lines first described, 5 other OP-derived cell lines (OP1, OP4-17, OP4-18, OP5-1 and OP5-C) from an earlier attempt to isolate OP cell lines

(Boolay & Illing, unpublished data) were included. The GT1-7 cell line (Mellon *et al.*1990; Table 2.1) was used as a positive control for markers such as GnRH and Olf-1 (Illing & Hapgood unpublished data). The COS-1 cell line, derived from monkey kidney cells (Gluzman 1981; Table 2.1), served as a negative control.

3.3.2 Cell selection using RT-PCR

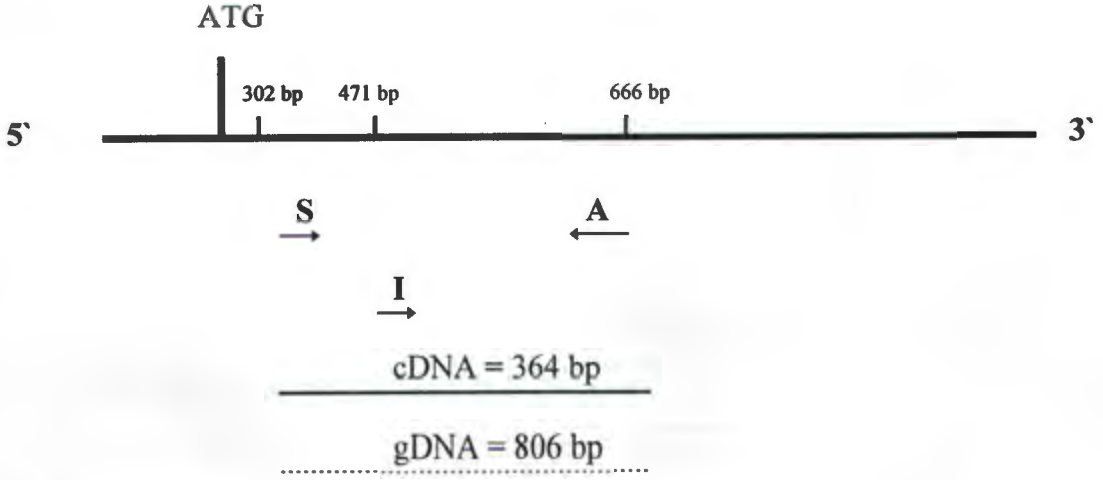
The aim of this screen served as an initial characterisation of all the cell lines and a means to select a few cell lines for further study. In order to select cells that were unique and that had the characteristics of cells present in the olfactory epithelium, I chose a number of markers to identify the cells as progenitor or stem cells, olfactory neuronal cells and/or olfactory supporting cells. These markers included: Pax-6, nestin, Olf-1, BF-1, Mash-1, $p75^{NGFR}$, GnRH and olfactory receptor transcripts. Primers were designed for use in reverse transcription polymerase chain reaction (RT-PCR) (Table 2.4). The OP cells were grown at 33°C in DM-10 till ~ 80% confluency, harvested with PBS containing trypsin and EDTA, resuspended in DM-10 and then pelleted. The cell pellets were then used directly to isolate total RNA or stored at -70°C till required for RNA extraction. Total RNA was isolated from the cells, reverse transcribed and the specific DNA fragments amplified using the polymerase chain reaction with primers designed to anneal to the 5' and the 3' sequences in the genes of interest. PCR-amplified reverse transcripts of total cellular RNA were electrophoresed on agarose gels alongside a 100 bp DNA ladder marker. With the use of β-actin primers as an internal control all the cell lines yielded, in relatively equal amounts the expected size transcript of 364 bp for cDNA [Fig 3.2 (B)]. Importantly, none of the cell lines amplified an 806 bp product, which would have been expected had genomic DNA been amplified (E. Vreugenheid personnel communication). This was important to ensure that none of the RT-PCR results would be incorrectly interpreted and indicated that the RNA was not contaminated with genomic DNA. In all cases the water control, in the absence of cDNA, was negative.

3.3.2.1 Olf-1 RT-PCR

The majority of the cell lines were positive for Olf-1 expression (GT1-7, OP1, OP4-17, OP4-18, 3T3, OP6, OP8-OP31, OP34-OP36, OP38-OP42, OP47-OP51, OP53, OP55, OP56, OP58 and OP59) while (OP5-1, OP5-C, COS-1, OP7, OP32, OP37, OP43-OP46, OP57) lacked any Olf-1 expression [Fig 3.3 (B)]. The PCR results were confirmed by internal oligonucleotide hybridisation.

β-actin primers

A



B

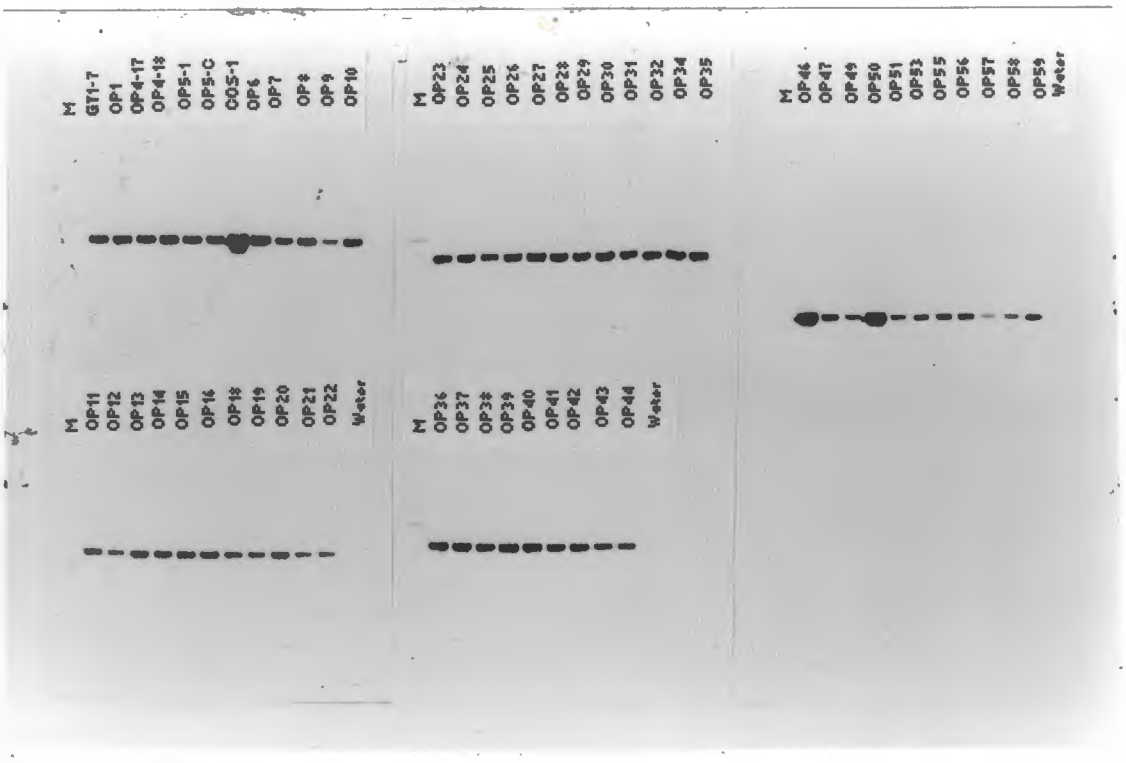
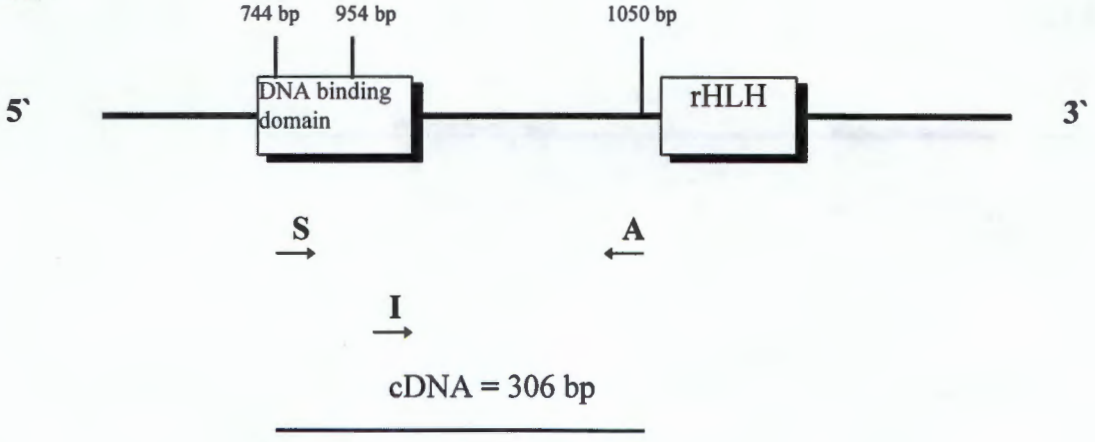


Fig 3.2 **(A)** Schematic diagram indicating the position of the sense 'S', antisense 'A' and the internal 'I' primers for β-actin. Amplification of cDNA resulted in a 364 bp product (arrows) while amplification of genomic DNA (gDNA) resulted in a 806 bp product (E. Vreugenheid). **(B)** All the cell lines amplified a cDNA PCR product of 364 bp but non amplified any gDNA product. The water control in the absence of any cDNA was clean of any contamination.

Olf-1 Primers

A



B

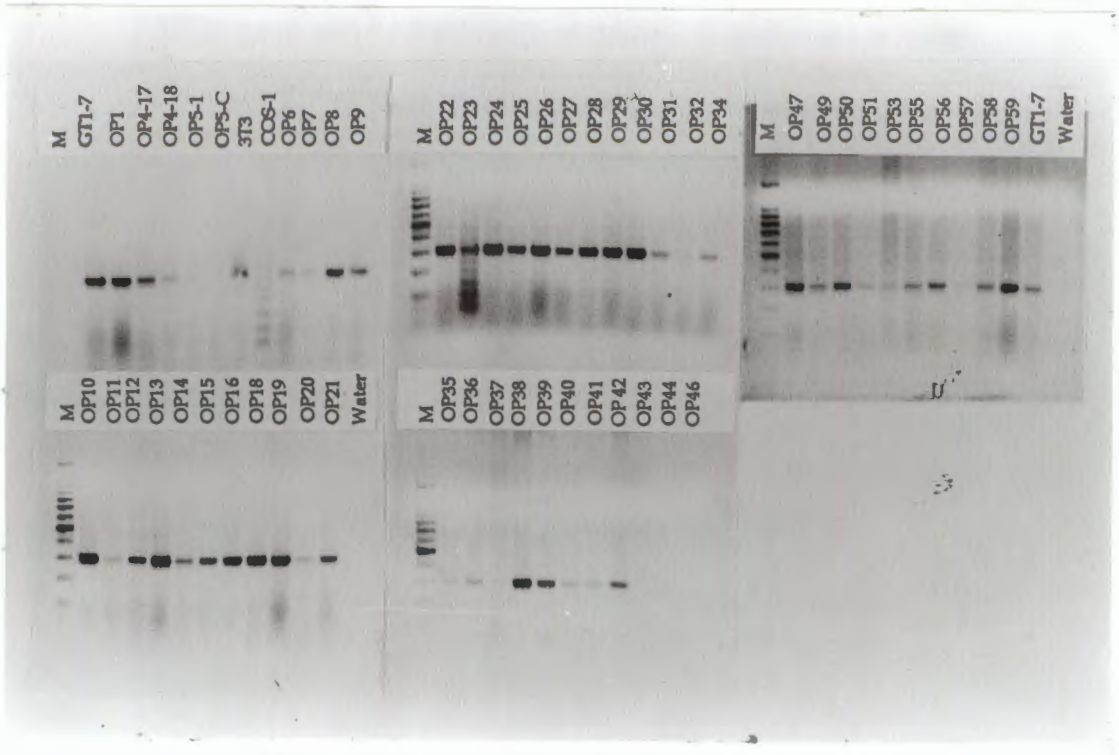


Fig 3.3 **(A)** Schematic diagram of Olf-1 primers indicating the position of the sense 'S', antisense 'A' and internal 'I' primers. The rHLH dimerisation domain, consists of a conserved rHLH domain with repeated α-helices (Hagman *et al.* 1993; Wang *et al.* 1997). The DNA binding domain contains a novel zinc domain, which is important for DNA recognition. **(B)** Reverse-transcribed cDNA from the OP cell lines was amplified with the 'S' and the 'A' primers. The positive cell lines amplified a PCR product of 306 bp (arrows) which was absent in both the negative cell lines as well as in the water control. The marker on the left hand side of the gel is a Promega 100 bp DNA ladder; 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp

3.3.2.2 BF-1 RT-PCR

BF-1 expression was detected in OP1, OP4-17, OP5-1, OP6-OP13, OP15, OP18-OP27, OP29, OP31, OP32, OP34-OP38, OP41-OP44, OP47-OP50, OP53, OP56 cell lines [Fig 3.4 (B)]. To confirm these results, the PCR products were transferred to nylon membranes and hybridised to a randomly labelled cDNA probe (SmaI digest of cDNA to BF-1; Table 2.3) (data not shown) which extended across the region amplified by the BF-1 primers (Table 2.4). These results suggest that most of the cell lines isolated have the potential to be proliferating neuronal progenitor cells from the olfactory neuroepithelium or the telencephalon.

3.3.2.3 GnRH RT-PCR

PCR transcripts for the positive GnRH cell lines were very weak or absent but were intensified when hybridised to a GnRH internal oligonucleotide [Fig 3.5 (B)]. The positive cell lines were GT1-7, OP4-17, OP4-18, OP5-1, OP6, OP8, OP9-OP16, OP18, OP19, OP21, OP36-OP43, OP47, OP50, OP51, OP53, OP55, OP56, OP59. The remaining cells were negative. The primers for GnRH were designed across two introns. Amplification of genomic DNA would have resulted in a product of 3 kb - much larger than the expected cDNA transcript size of 241 bp. None of the cell lines amplified a genomic DNA product.

3.3.2.4 Olfactory receptor transcript RT-PCR

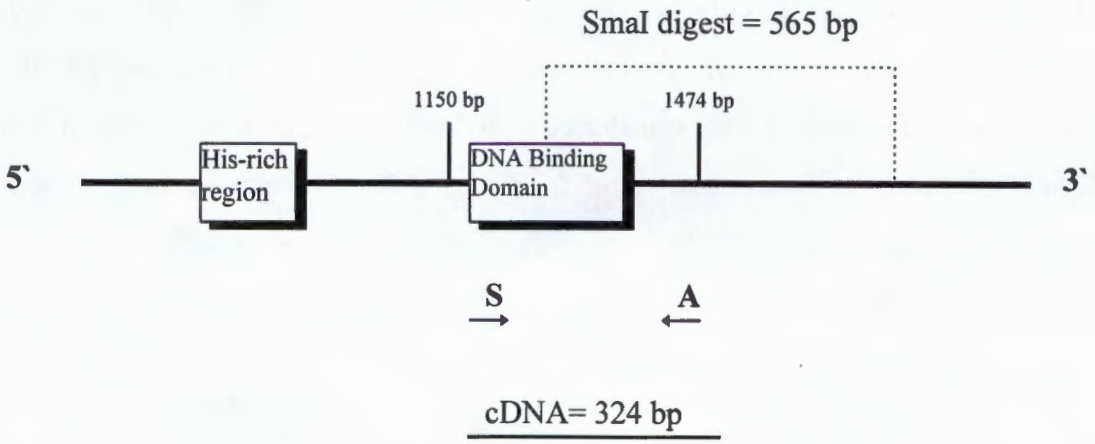
No PCR product that corresponded to odorant receptor transcripts could be amplified from cDNAs obtained from any of the cell lines when grown at 33°C [Fig 3.6 (B)]. Genomic DNA, used to optimise the PCR primers, gave the expected strong and sharp PCR product of 510 bp [Fig 4.4 (G)]. Genomic DNA was used as a control, as the genes for olfactory receptor neurons lack introns.

3.3.2.5 Nestin RT-PCR

Primers designed to amplify a 252 bp product across the α-helix domain of nestin cDNA (Kachinsky *et al.* 1994) yielded a faint transcript in OP38 only. When, however, the nested 5' nestin primer I203 was used together with the previous reverse primer, a 199 bp product was observed in

BF-1 Primers

A



B

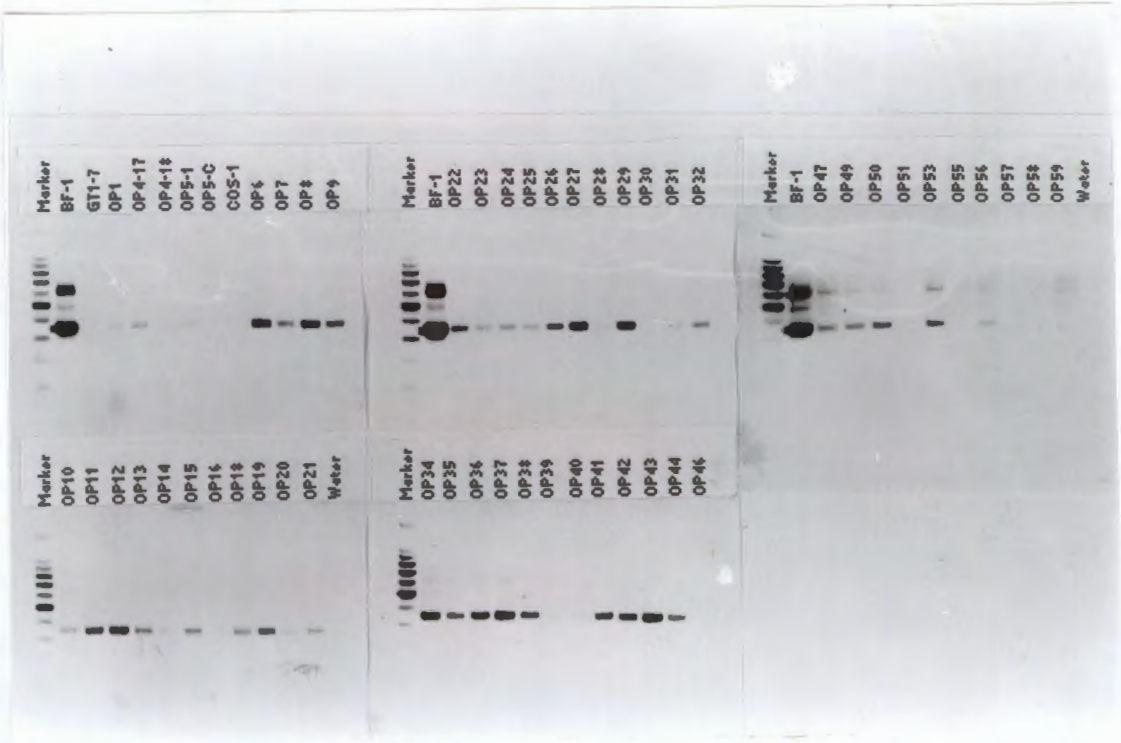
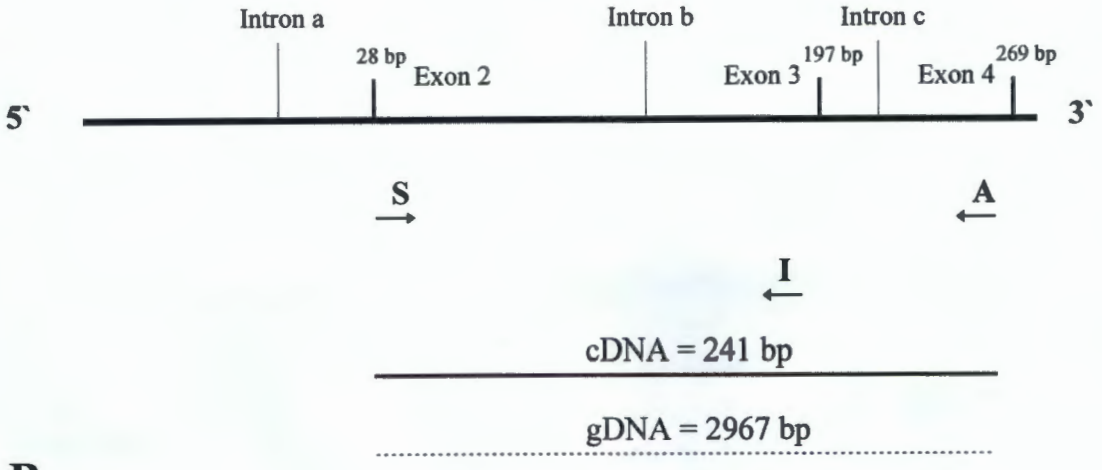


Fig 3.4 **(A)** Schematic diagram of BF-1 primers designed across the DNA binding domain ('forkhead' domain). The sense 'S' and the antisense 'A' primers amplified a cDNA fragment of 324 bp. To confirm the PCR results, a Sma I cDNA fragment of BF-1 was excised (565 bp) and hybridised to the PCR product. **(B)** cDNA to the OP cell lines were amplified with the 'S' and the 'A' primers identifying the positive BF-1 cell lines with a 324 bp PCR product. This 324 bp PCR product was absent in the negative water control as well as the COS-1 cell line. As a positive control, cDNA to BF-1 was amplified, resulting in three transcripts of 650 bp, 500 bp and 324 bp. The expected size of 324 bp was the strongest transcript.

GnRH Primers

A



B

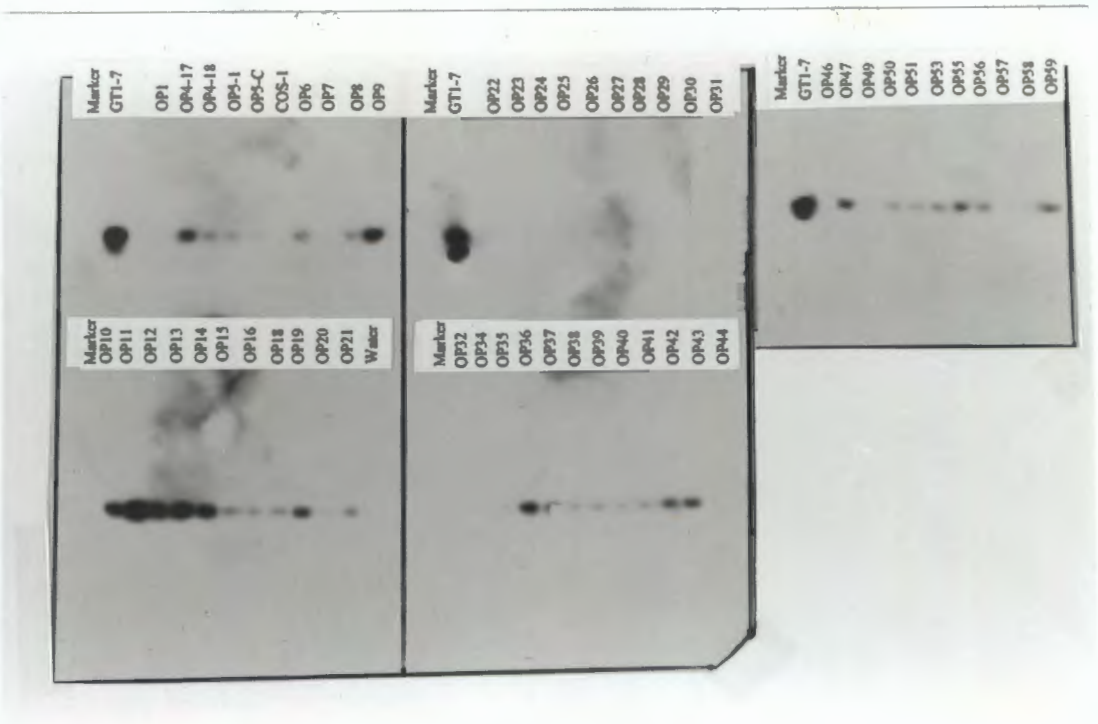
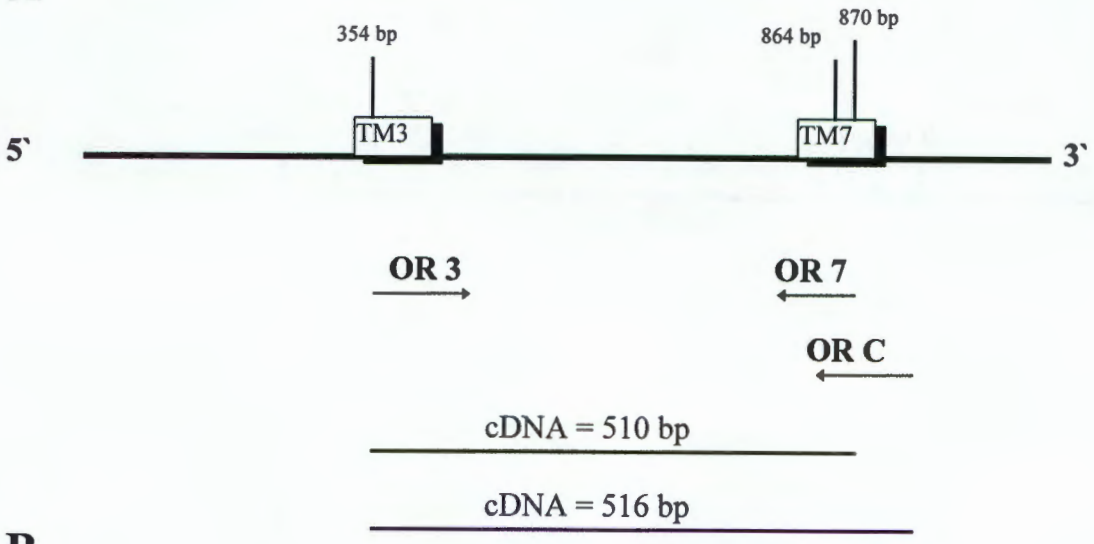


Fig 3.5 **(A)** Schematic diagram of the GnRH protein indicating the positions of the three introns (Introns a, b and c) and the four exons (Exon 1, 2, 3 and 4) (Zhen *et al.* 1997). The sense 'S' primer in exon 2 and the antisense 'A' primer in exon 4 were designed across introns b and c. Amplification of cDNA to GnRH resulted in a 241 bp product which was smaller than the ~3 kb product expected for gDNA amplification. The internal primer 'I' sequence was in exon 3. **(B)** The OP cell lines cDNA were amplified with 'S' and 'A' primers. The majority of the cell lines were negative or faintly positive for a 241 bp product. The PCR products were transferred to nylon membrane and hybridised to the end labelled 'I' oligonucleotide. The cell lines which were faintly positive on the agarose gel hybridised strongly to 'I' and some negative cell lines hybridised weakly to the 'I' oligo (arrows). The water control was negative. The positive control was GT1-7 cell line, which is a hypothalamic cell line that express GnRH (Mellon *et al.* 1990).

Olfactory receptor transcript primers

A



B

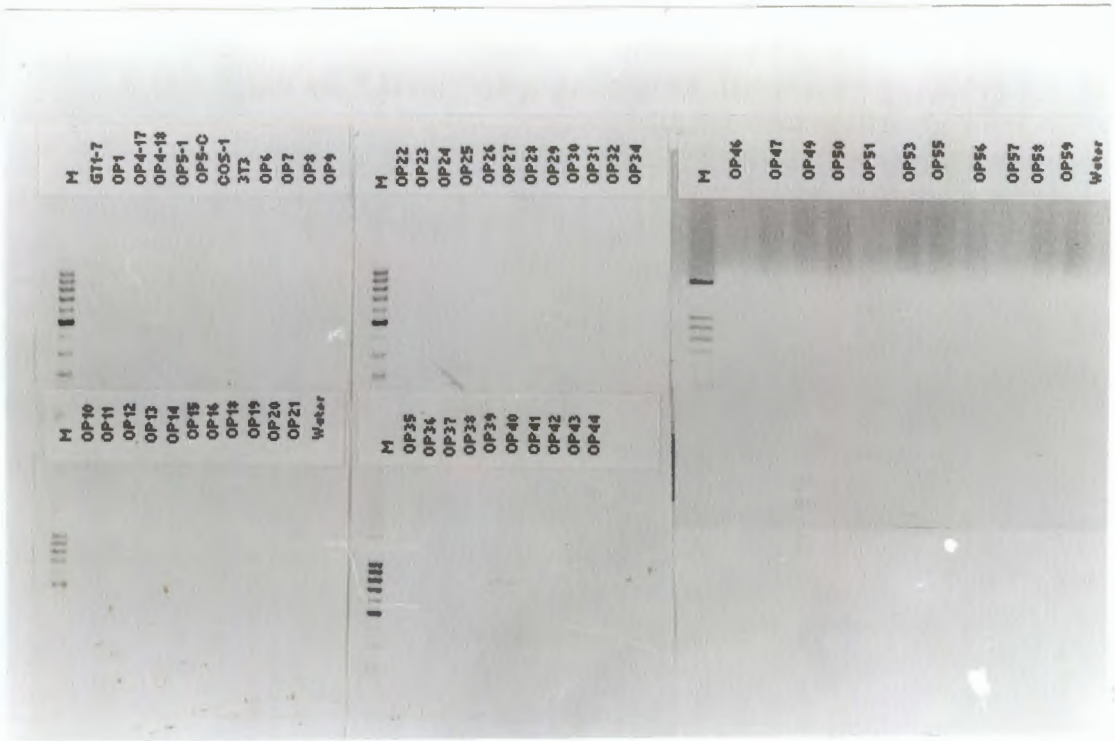
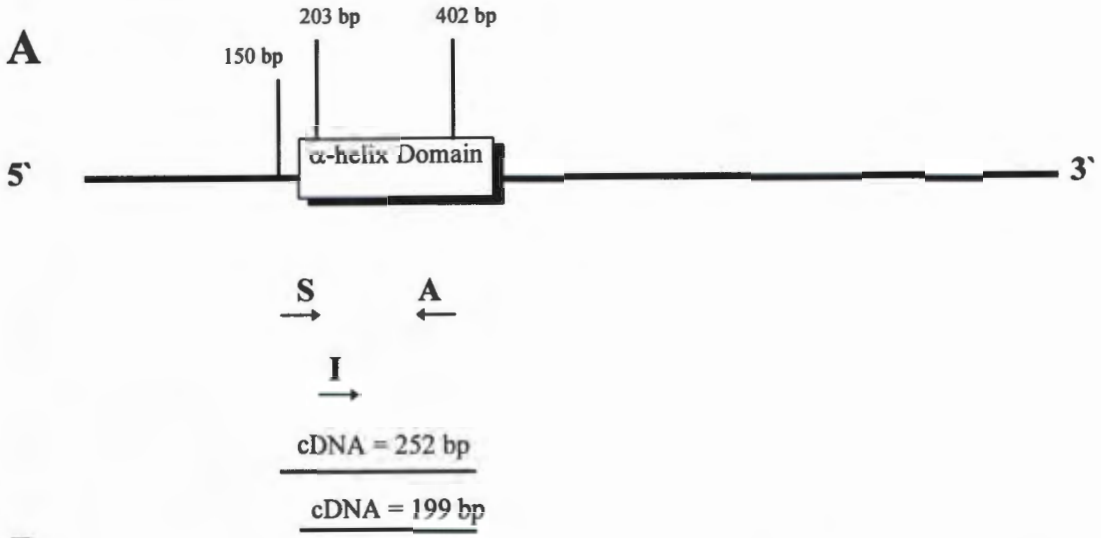


Fig 3.6 **(A)** Schematic diagram of olfactory receptor transcript primers. The primers corresponded to transmembrane (TM) 3 for the sense primer, OR 3 and TM 7 for the antisense primers, OR 7 and OR C. OR 7 and OR C were overlapping primers with different 3' ends (Table 2.4). For amplification of cDNA the primers OR 3 and OR 7 were used (510 bp). For cloning (Chapter 5), the primers OR 3 and OR C were used (516 bp) as their 3' ends were more compatible for cloning into the pGEM T-vector. **(B)** Agarose gels to the cDNA of the OP cell lines which were amplified with primers OR 3 and OR 7. None of the cell lines amplified the expected PCR product of 510 bp at 33°C in DM-10.

Nestin primers



B

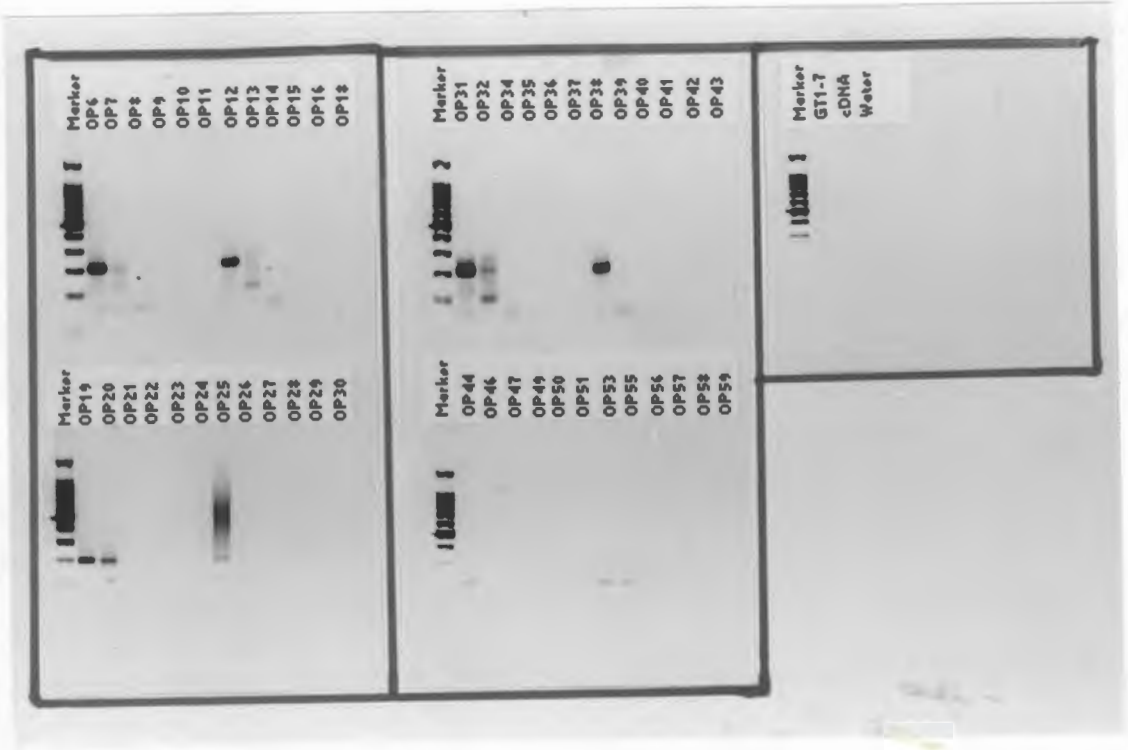


Fig 3.7 **(A)** Schematic diagram of nestin primers representing the position of the sense 'S' and the antisense 'A' primers, which were designed to span the α-helix domain to amplify a PCR product of 252 bp. An internal sense 'I' oligo was used in nested RT-PCR with the 'A' primer to yield a product of 199 bp. **(B)** An agarose gel of nested nestin RT-PCR with 'I' and 'A' primers. The positive cell lines were identified by an expected 199 bp transcript. Nested RT-PCR was performed on the RT-PCR with primer pair 'S' and 'A' to enhance the amplified product which was very faint.

cell lines; OP6, OP12, OP19, OP20, OP25, OP31, OP32, OP38, OP44, OP53 and OP55 [Fig 3.7 (B)]. The remaining cell lines were completely negative, including the negative water control.

3.3.2.6 Pax6 RT-PCR

The Pax6 primers; Pax-6.43 F and Pax-6.44 R (Table 2.4; A. Chess, personal communication) were designed to amplify a 736 bp product. RT-PCR of cDNA using this primer pair showed that only four cell lines (OP27, OP34, OP37, OP47) expressed the Pax6 transcript. These results were confirmed by hybridisation with the internal oligonucleotide, Pax-6 I (Table 2.4) to the PCR products and in addition five cell lines (OP29, OP36, OP44, OP49 and OP59) [Fig 3.8 (B)], that were previously negative now appeared positive on the autoradiograph. The very strong hybrid signal was a result of probing the PCR reaction which used the Pax6 cDNA as a substrate, for a positive control [Fig 3.8 (B)].

3.3.2.7 $p75^{NGFR}$ RT-PCR

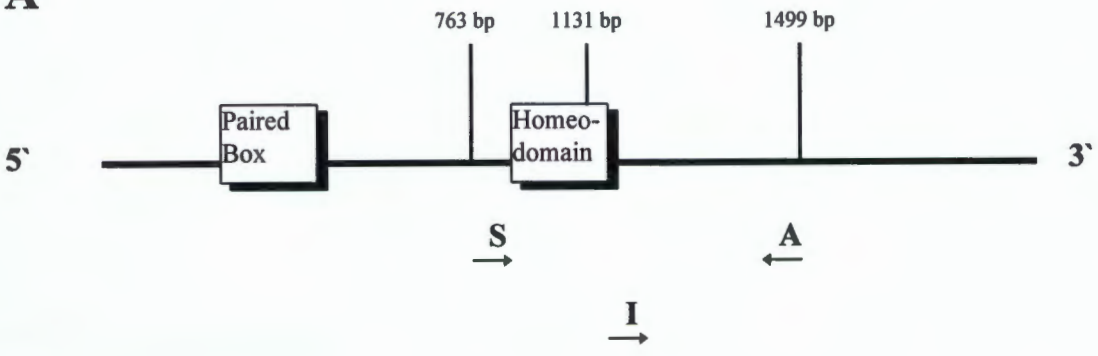
None of the cell lines tested (with the exception of OP13) expressed the expected 841 bp transcript for $p75^{NGFR}$ but a transcript was detected in the neuronal cell line GT1-7. Some cell lines of neuronal origin have been shown to express this marker which is restricted to the olfactory glial lineage (J. Roskams, personal communication). Furthermore, this primer pair for $p75^{NGFR}$ was optimised using total mouse brain cDNA when it amplified the expected PCR product of 841 bp. However, when the primers were used on the OP cell lines, the positive cell line, OP13 amplified a band of 841 bp and a few additional bands of less than 550 bp. These smaller bands were also detected in a few of the negative cell lines [Fig 3.9 (B)]. To further analyse these results, genomic DNA was amplified using these primers. The product size with genomic DNA was ≥ 1kb. This confirmed that the transcripts obtained were not due to genomic DNA contamination (data not shown). The same results were obtained when the experiment was repeated.

3.3.2.8 Mash-1 RT-PCR

The primers to Mash-1 were designed to amplify the conserved bHLH domain, so generating PCR product of 451 bp (Calof *et al.* 1996; Table 2.4). In none of the OP cell lines was there a detectable transcript for Mash-1, either by direct analysis or by hybridisation to an end-labelled oligonucleotide.

Pax6 Primers

A



cDNA = 736 bp

B

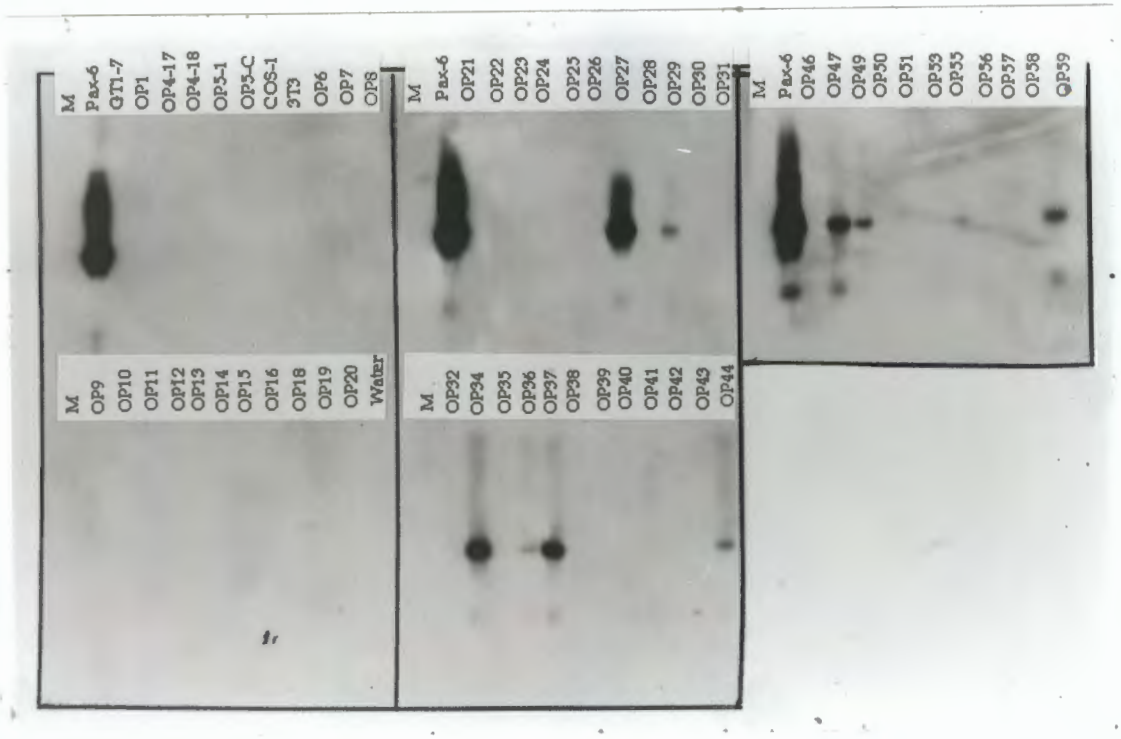
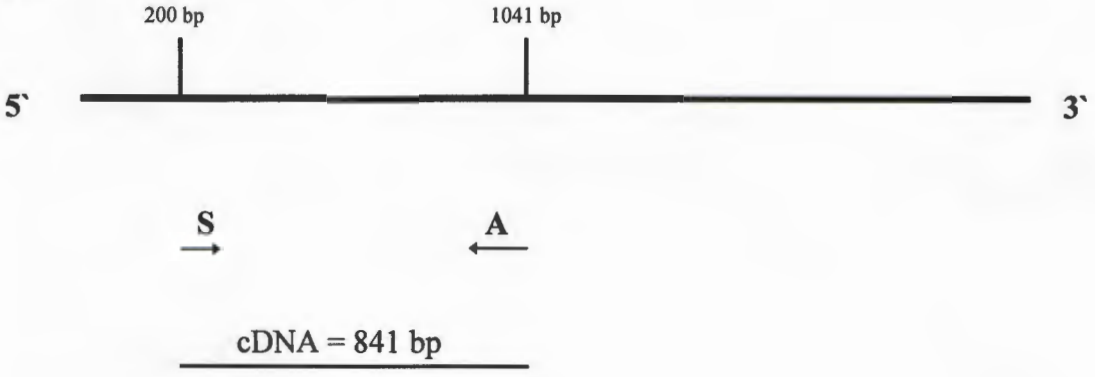


Fig 3.8 **(A)** Schematic diagram of Pax6 cDNA indicating the highly conserved paired domain of 128 amino acids and the homeodomain which contained a helix-loop-helix (HLH) motif. The 'S' and the 'A' primers were designed across the homeodomain to yield a cDNA PCR product of 736 bp. A sense oligonucleotide was designed as an internal 'I' probe for Southern blotting. **(B)** An autoradiograph exposure of Pax6 RT-PCR ('S' and 'A' primers) hybridised with the 'I' probe. The expected size PCR transcript of 736 bp was seen in the positive control, Pax6 cDNA, as well as the positive cell lines (arrows). The cell lines which were weakly positive on the agarose gels were stronger on the autoradiograph and some negative cell lines now appeared faintly positive. The Pax6 cDNA amplified a strong, sharp 736 bp product, which hybridised strongly to the 'I' probe resulting in a smear in the Pax6 lane.

p75^{NGFR} Primers

A



B

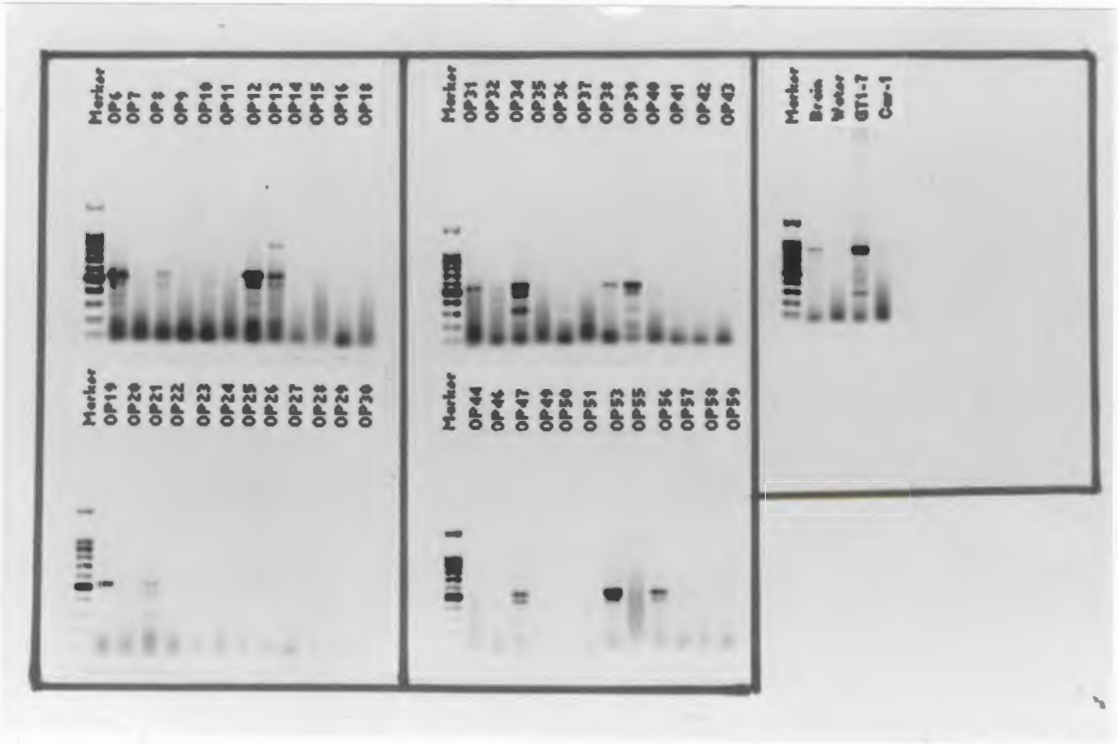
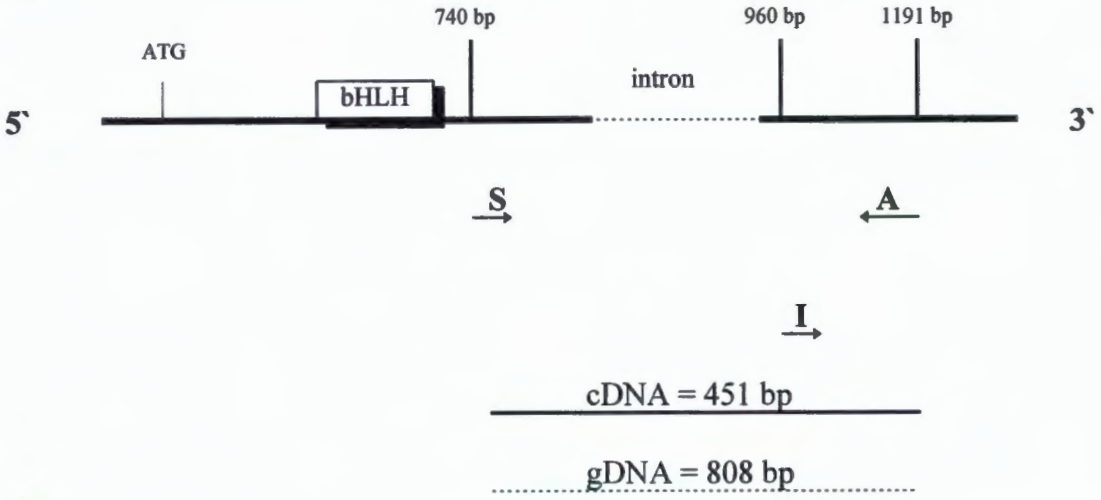


Fig 3.9 **(A)** Schematic diagram of p75 low affinity nerve growth factor receptor primers (p75^{NGFR}). The sense 'S' and antisense 'A' primers were designed to amplify a PCR product of 841 bp of rat p75^{NGFR}. **(B)** Agarose gels representing the RT-PCR products of the OP cell lines amplified with the 'S' and 'A' primers. The positive control, total mouse brain cDNA and the OP13 and GT1-7 cell lines were positive. The other cell lines were negative. While some of these negative cell lines were completely negative, other negative cell lines amplified smaller PCR transcripts. The water control was clean except for the primer dimers.

Mash-1 Primers

A



B

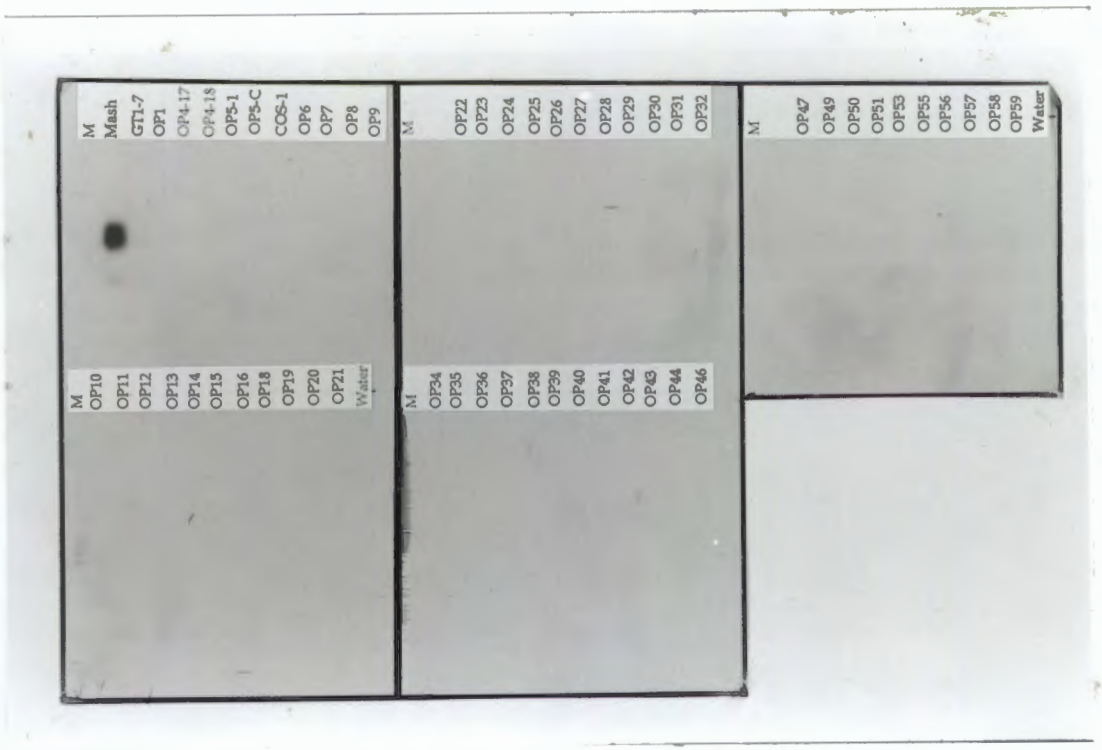


Fig 3.10 **(A)** Schematic diagram of Mash-1 primers indicating the positions of the highly conserved bHLH DNA binding domain and the sense 'S' and the antisense 'A' primers, which amplified a cDNA product of 451 bp but in the presence of gDNA, amplified a product size of 808 bp. A sense primer was designed as an internal 'I' probe for Southern blotting. **(B)** An autoradiograph of the PCR products which were transferred to nylon membrane and hybridised with an end labelled internal 'I' probe. No Mash-1 transcripts were detected. The positive control, cDNA to Mash-1, gave the expected 451 bp fragment which hybridised to the 'I' probe. None of the cell lines amplified a 808 bp PCR product, confirming the absence of any contaminating genomic DNA.

The positive control, Mash-1 cDNA, gave the expected product of 451 bp. In addition, none of the cell lines amplified a genomic PCR product of 808 bp, confirming the absence of contaminating genomic DNA [Fig 3.10 (B)].

On the basis of these results, four cell lines (OP6, OP27, OP47 and OP55) which expressed different combinations of these markers were chosen for further characterisation. (Table 3.1).

Table 3.1 *RT-PCR phenotypes of cell lines at 33°C in DM-10. The presence (+) or the absence (-) of a PCR product is indicated.*

Cell line	β-actin	Olf-1	BF-1	GnRH	Nestin	ORN	Pax-6	Mash-1	p75
OP6	+	+	+	+	+	-	-	-	-
OP27	+	+	+	-	-	-	+	-	-
OP47	+	+	+	+	-	-	+	-	-
OP55	+	+	-	+	+	-	-	-	-

3.3.3 *Growth curves of cell lines grown at 33°C and at 39°C*

3.3.3.1 Population doubling time (PDT)

The population doubling time (PDT) is defined as the time, in hours, taken for the cell number to double (David 1994). The population doubling times of the OP cells grown at 33°C in DM-10 were calculated from the linear regions of semi-log plots of replicate cell counts as a function of time. The cells were maintained in culture in duplicate for a period of 20 days, harvested and counted every alternate day. The linear regions were selected visually to exclude the lag phase and the tailing off of growth with confluency. The doubling times were as follows: OP6 - 99 hours, OP27 - 52 hours, OP47 - 31 hours and OP55 - 60 hours [Fig 3.11 (A-D); Table 3.2].

The saturation density of a cell line is defined as that number of cells present in a culture when no rise in cell counts is seen (Brugge & Butel 1975) and is also referred to as the plateau stage, where no net increase in cell number occurs (David 1994). For the OP cells at 33°C, the saturation densities were calculated as follows: For OP6 - 6.1 x 10^5 cells/cm^2, OP27 - 3.0 x 10^5 cells/cm^2, OP47 - 2.4 x 10^5 cells/cm^2 and OP55 - 7.9 x 10^5 cells/cm^2.

Table 3.2 *Population doubling times (PDT) of each cell line in DM-10*

The PDT were calculated on a semi-log graph for cells maintained at 33°C only (33°C) or cells maintained at 33°C after being grown at 39°C for a period of 8 days (39°C → 33°C) (Materials and methods sections 2.3.5.2 and 2.3.5.3). The time period used to calculate the PDT is indicated in hours.

Cell line	33°C			39°C → 33°C		
	PDT (hours)	Time Period (hours)	Correlation coefficient	PDT (hours)	Time Period (hours)	Correlation coefficient
OP6	99	120-384	0.98	52	384-504	0.95
OP27	52	24-264	0.98	31	336-432	1
OP47	31	72-168	0.99	46	336-432	0.99
OP55	60	72-264	0.96	47	336-432	0.99

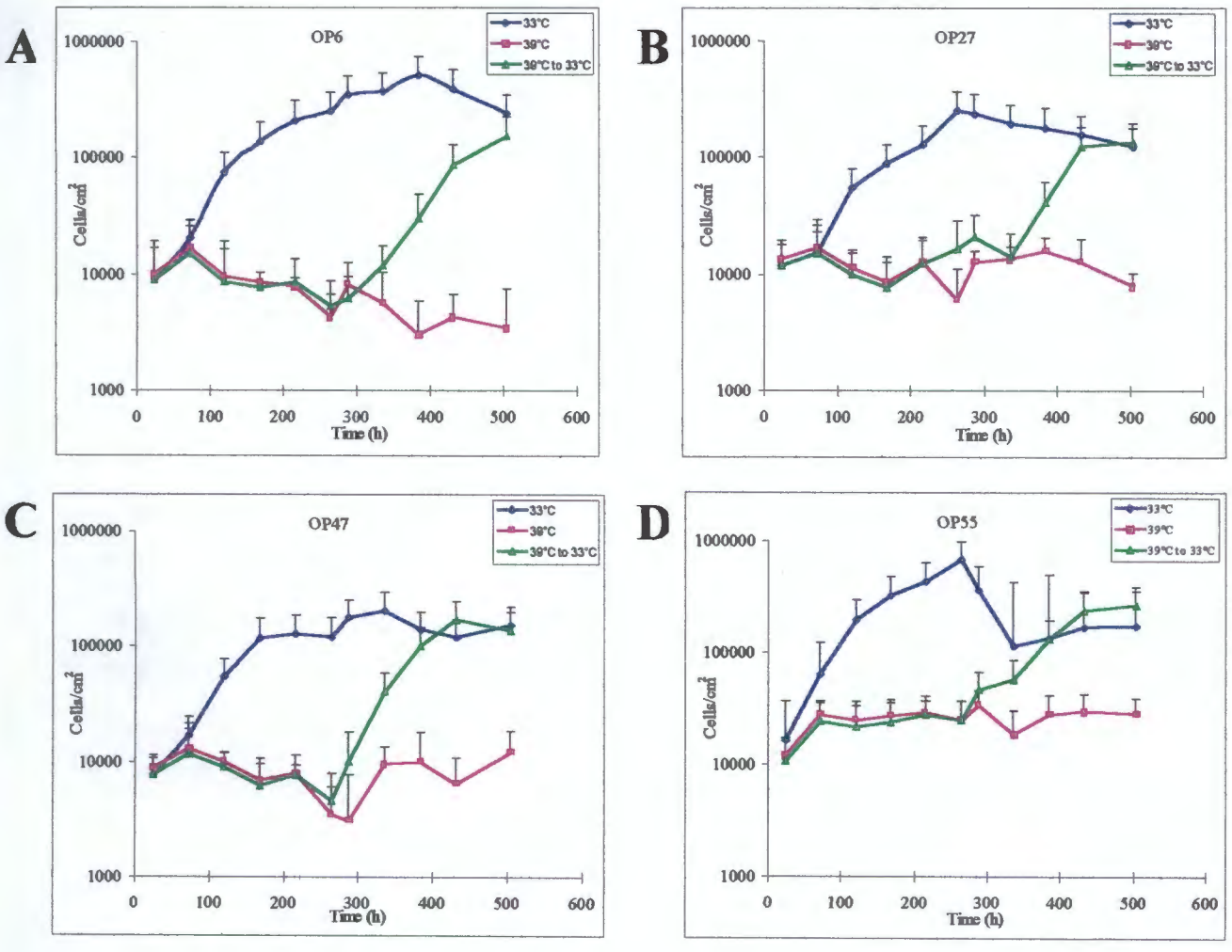


Fig 3.11 Growth curves of OP6 (A), OP27 (B), OP47 (C) and OP55 (D) cell lines performed at 33°C, 39°C and when cells were maintained at 39°C for 8 days (192h) and then shifted down to the permissive temperature of 33°C to determine whether the inactivation of the SV40 Tag at 39°C was reversible. Cells were maintained in DM-10, harvested and counted in equal volumes of trypan blue, a vital dye.

To determine the growth of cells at 39°C, the OP cells were plated out in DM-10, allowed to settle at 33°C for 24 hours and then shifted to 39°C. Cells were harvested and counted on alternate days. When shifted to 39°C the OP cells divided approximately once and remained at a relatively constant density thereafter. The OP6 cells did not survive well at 39°C beyond 300 hours after which they decreased significantly in number and looked very unhealthy. The OP55 cells grown at 39°C grew better and looked healthier than the other OP cell lines. OP47 and OP55 maintained relatively constant cell numbers over 500 hours.

As a control to study the effect of growth of the OP cell lines at the two different temperatures, the 3T3 murine fibroblast cell line was grown in parallel at 33°C and at 39°C. The cells grew well at 33°C and, when shifted to 39°C, they flourished (data not shown). Since 3T3 cells grew considerably faster at 39°C, this implied that the elevated temperature was optimal for growth of mouse derived cell lines.

3.3.3.2 Reversion of Growth

To demonstrate the reversibility of growth of the OP cells under restrictive conditions of 39°C, a shift down experiment was performed in which OP cells were maintained at 39°C for 10 days in DM-10 and then shifted back to the permissive temperature of 33°C for a further 10 days. The OP cells were harvested and counted every alternate day.

All four OP cell lines studied showed that reversion took place [Fig 3.11 (A, B, C, D) Table 3.1]. Once shifted to 33°C, the OP cells re-entered the cell cycle after an initial lag period with a different PDT to the same cells maintained only at 33°C. The PDT was calculated on a semi-log graphs and is as follows: OP6 has a PDT of 52 hours with a correlation coefficient of 0.95, OP27 has a PDT of 31 hours and a correlation coefficient of 1, OP47 has a PDT of 46 hours and a correlation coefficient of 0.99, and OP55 has a PDT of 47 and a correlation coefficient of 0.99.

These results were very encouraging as they showed that the inability of the cells to grow at 39°C was not due to the elevated temperature but rather due to inactivation of the SV40 Tag at 39°C.

3.3.4 *Clonality and analysis of proviral DNA*

The site of integration of the retrovirus carrying the SV40 Tag was checked to confirm that the cell lines were of clonal origin. Chromosomal DNA isolated from the OP cells was digested with BglII which cuts once within the genome of the retrovirus [Fig 3.12 (A)] and extensively within the host DNA.

If a cell line comprised a mixture of cell types derived from different retroviral insertions, one would have expected multiple bands on the Southern blot. Southern blot analysis [Fig 3.12 (B)] showed that in all cases the probe hybridised to a well defined single band of sizes 3.7 kb, 6.1 kb, 7.0 kb, 3.8 kb, 7.0 kb and 5.1 kb for cell lines OP4-18, OP5-1, OP6, OP27, OP47 AND OP55 respectively. This shows that these cell lines are clonal. As expected, the random nature of the retroviral insertion site and the situation of the site in relationship to neighbouring BglII restriction sites generated hybridising fragments that differed in size from one line to another. The negative control cell line, COS-1 (Gluzman *et al.* 1981) did not hybridise to the probe since it was immortalised with the SV40 retrovirus lacking the neomycin resistance gene.

3.3.5 *Expression of SV40 protein at 33°C and 39°C*

The OP cell lines were immortalised with a temperature-sensitive SV40 Tag mutant so that the SV40 Tag should be expressed and active at the permissive temperature of 33°C, but inactive and rapidly degraded at the non-permissive temperature of 39°C (Tegtmeyer 1975). Osborn & Weber (1975) found that, after 3 days at the non-permissive temperature at least 15% of the cells still expressed the SV40 Tag so that data obtained during this period should be interpreted with caution and in the knowledge that the SV40 Tag might still be active. To establish the effects of a temperature shift on the expression of SV40 Tag in the OP cells I grew the cells to 70-80% confluency at the permissive temperature and then for a further period of 10 days at 39°C. The cells were then fixed and examined immunohistochemically for SV40 Tag expression. As shown in Fig 3.13, all the cells maintained at 33°C were SV40 Tag positive. After 10 days at 39°C SV40 Tag could no longer be detected.

3.3.6 *BrdU incorporation - cell division at 33°C and 39°C*

Growth curves [Figs 3.11 (A, B, C, D)] showed that the OP cells maintained at 33°C proliferated continuously, whereas after 10 days at 39°C, cell number remained constant or decreased. In order

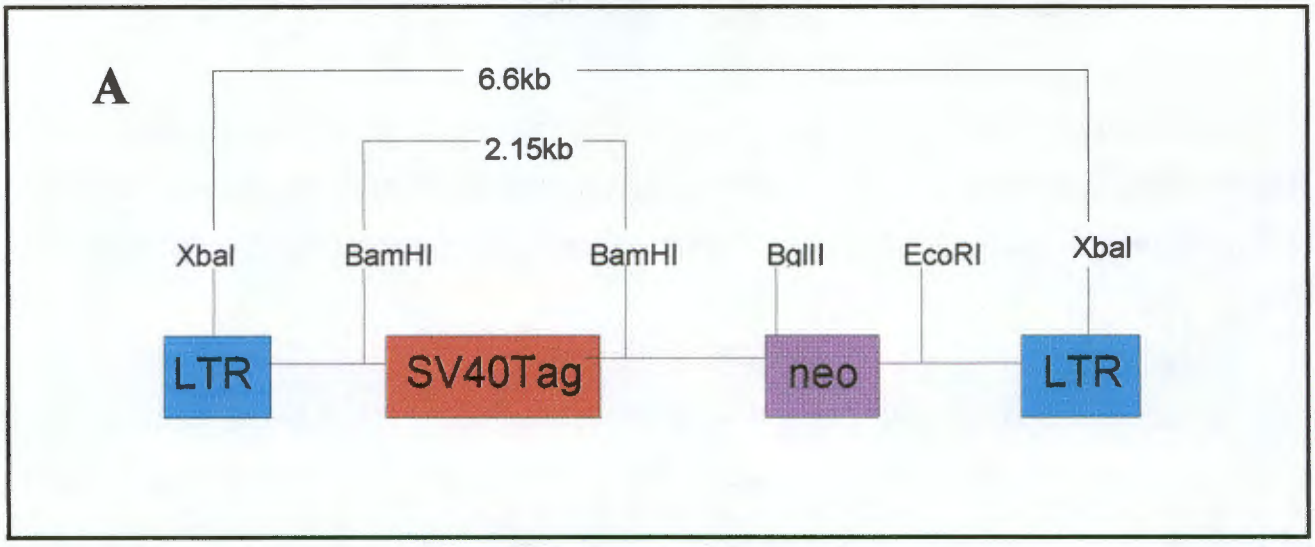


Fig 3.12 (A) Schematic representation of recombinant retroviral plasmid of pZip-Neo-SV40*U19tsA58* carrying both SV40 Tag mutations, namely the *U19* and the temperature sensitive *tsA58* mutation (Almazan & McKay 1992). The selectable marker gene neomycin resistance (neo) encodes a protein, when expressed in cells, confers resistance to the toxin G418. The SV40 Tag is expressed by the promoter and enhancer in the Moloney long terminal repeats (LTRs). XbaI, BamH1, BglII and EcoRI are restriction sites at which these enzymes digest within the retroviral genome.

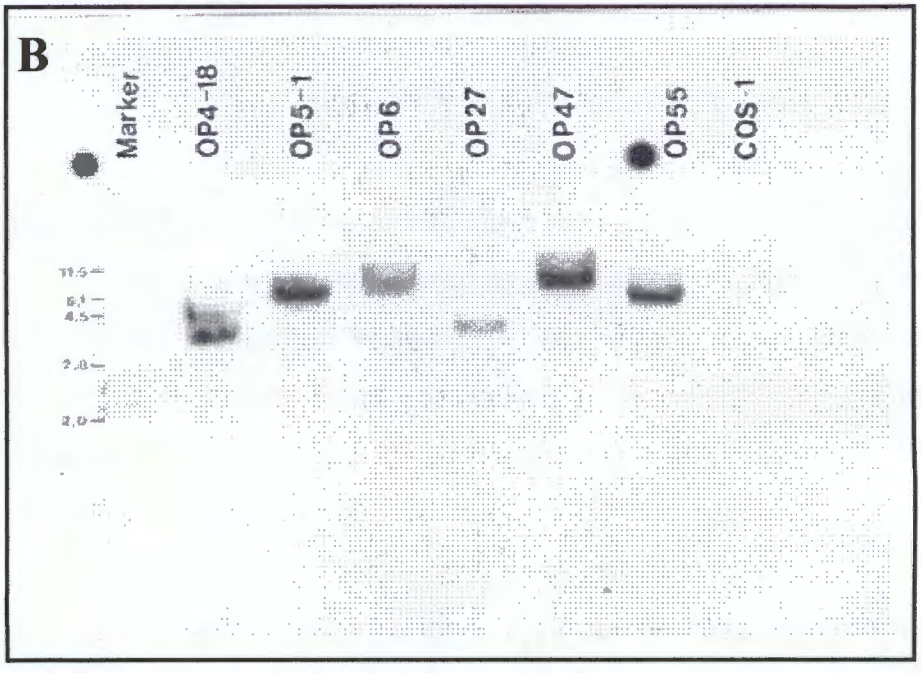


Fig 3.12 (B) Analysis of proviral DNA. Twenty micrograms of high molecular weight DNA prepared from the OP4-18, OP5-C, OP6, OP27, OP47, OP55 and COS-1 cells were digested with BglII restriction enzyme and transferred to nylon membranes. The Southern blot was hybridised with randomly primed ^{32}P-labelled neomycin resistant cDNA. Numbers on the left of the blot shows the molecular weights of the λ-PstI size marker in kb. COS-1 cell line, is immortalised with the SV40 Tag but lacks the neomycin gene, and serves as a negative control. The data showed that the cell lines carried a single viral insertion and were, therefore, clonal.

to check whether cells at 39°C were quiescent, or dividing and dying, I studied BrdU incorporation at 33°C and at 39°C. Cells were grown to 70-80% confluency at 33°C or for 10 days after a temperature shift to 39°C and exposed to BrdU for 2 hours (33°C) or 2 hours and overnight (39°C). Subsequently immunolabelling with a monoclonal antibody to BrdU showed (Fig 3.14) that the 33°C cultures were proliferating (i.e. more than 50% of the cells passed through S-phase during the 2 hour of BrdU exposure) whereas none of the cells in the 39°C cultures synthesised DNA.

Fig 3.13 Light microscopy showing immunolocalisation of the SV40 Tag in the nuclei of OP6 (A), OP27 (B), OP47 (C), OP55 (D) cells at 33°C or the absence of staining at 39°C (E-H). SV40 monoclonal antibody was detected with DAB substrate (brown precipitate in the cells nuclei) and counterstained with hematoxylin (blue staining in cells nuclei) at 39°C to allow visualisation of negative cells. Cells at 33°C were not counterstained with hematoxylin as it interfered with the positive SV40 Tag staining at 33°C. The negative controls (I-J) were in the absence of primary antibody to SV40 Tag. No staining was detected in these controls. Magnification 100x, brightfield photomicrographs.

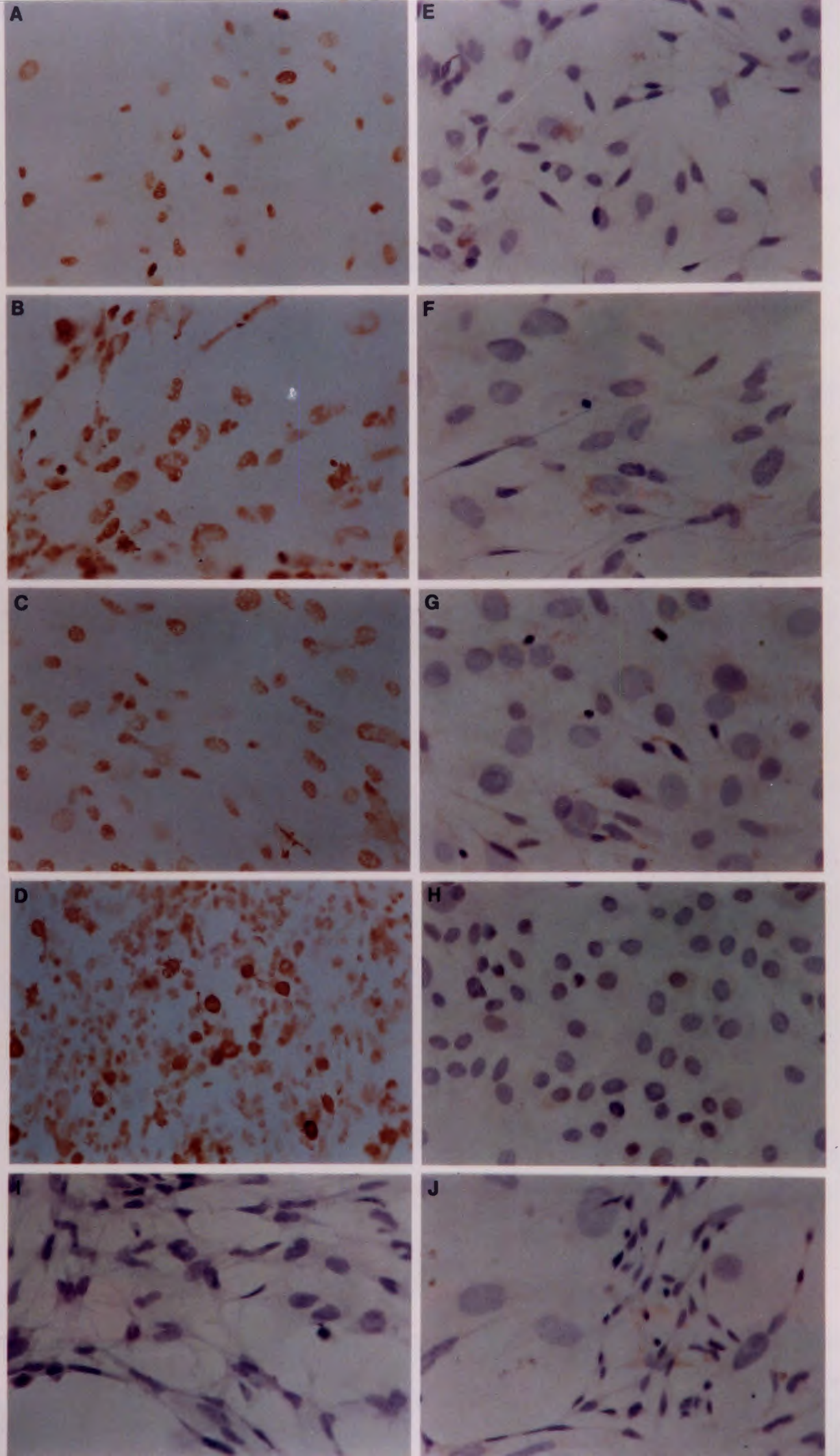
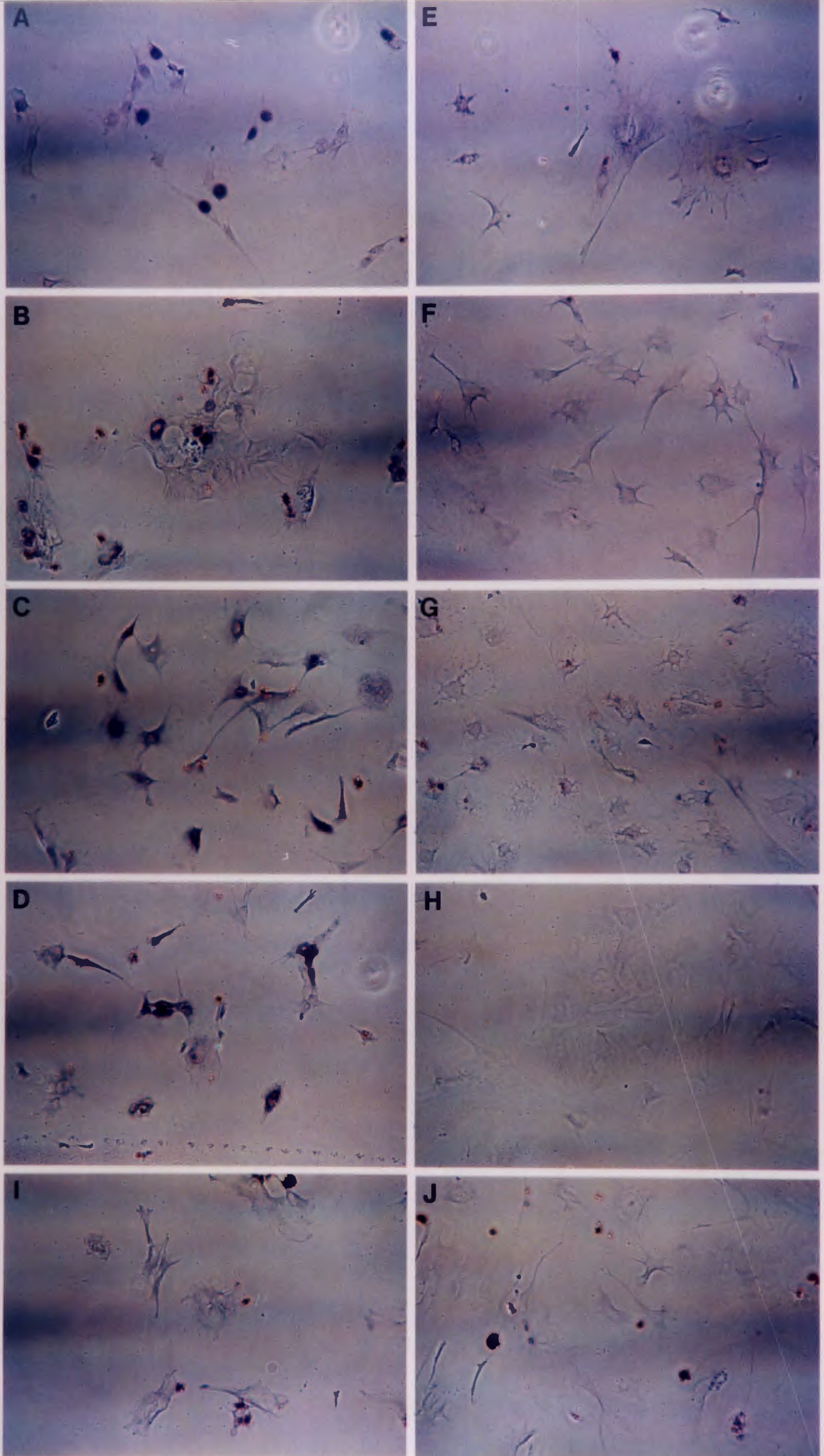

A
E
B
F
C
G
D
H
I
J

Fig 3.14 BrdU incorporation to determine whether cells maintained at 39°C undergo cell division. Phase contrast photomicrographs of cell lines: OP6 (A, E), OP27 (B, F), OP47 (C, G) and OP55 (D, H), grown in the presence of BrdU for 2 hours at 33°C (A-D) and 39°C (E-H). The presence of BrdU was detected with an antibody conjugated to alkaline phosphatase that resulted in a purple/blue precipitate in the nuclei of the positive cells at 33°C but all cells at 39°C were negative for BrdU, implying that no cell division took place within this time period. The negative controls (I-J) were performed as for the cells but in the absence of antibody to BrdU. No staining was detected in these slides. Magnification 50x.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3.3.7 *Soft agar assay - immortalisation vs. transformation*

Transformed cells, as apposed to those (such as 3T3 cells) that are immortal but not transformed, generally show anchorage-independent growth, proliferating in suspension or growing in colonies when dispersed in agar. When I seeded single cells from the OP lines in soft agar they remained so for 3 weeks, showing no signs of colony formation (Fig 3.15; Table 3.3). The K562 cells (Lozzio & Lozzio 1975) [Fig 3.15 (A)], on the other hand, formed colonies, at 7 days, with a frequency of 65-67%, while the 3T3 cells formed colonies at a frequency of 5% (Table 3.3).

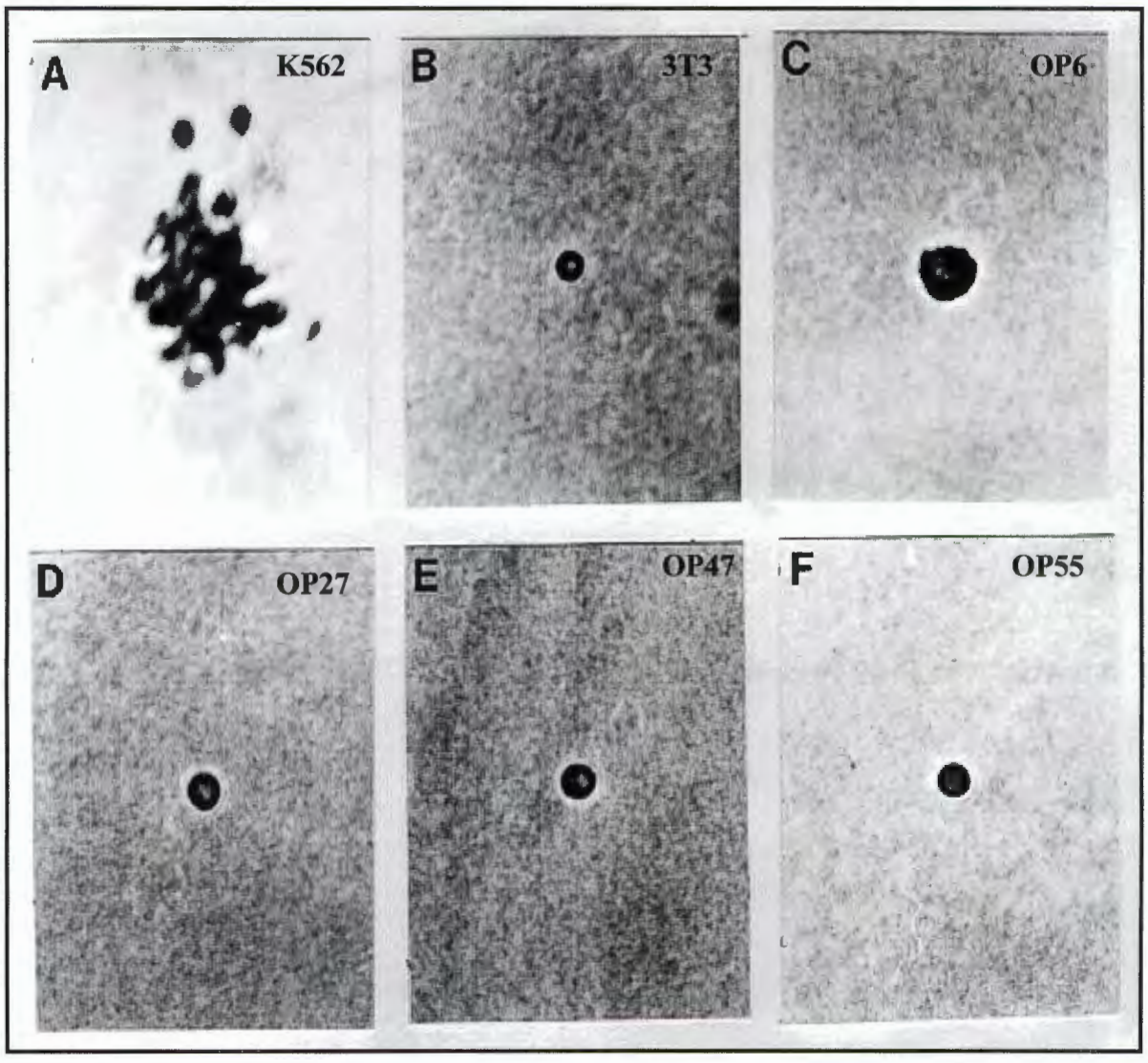


Fig 3.15 Soft agar colony formation in the OP cell lines. Cells were grown at 33°C in DM-10 in 1.32% Difco-bacto agar. As controls, the transformed K562 cell line and the 3T3 cell line were tested simultaneously at 37°C. Cells were maintained for 3 weeks in culture. The K562 cell line (A) formed colonies after one week, which continued to expand in size. The OP cell lines (C-F) and 3T3 (B) remained as single cells. This data showed that the OP6 (C), OP27 (D), OP47 (E), OP55 (F) has an immortalised phenotype rather than a transformed phenotype. Magnification = 16.25x

Table 3.3 *Quantitation of cells/colonies in soft agar assay.*

To determine whether the OP cell lines are transformed or immortalised, they were grown at 33°C in soft agar as described in methods (Section 2.3.6). As controls, the 3T3 cell line and the transformed cell line K562 (Lozzio & Lozzio 1975) was maintained in parallel at 37°C for a period of 21 days. Cells were grown in duplicate plates. With a 10x objective on an inverted Nikon microscope, 10 randomly chosen fields were selected and the number of colonies (C) and single (S) cells were counted. A percentage (%) was then calculated for each of the two plates. The results showed that the OP cell lines as well as the 3T3 cell line are immortalised but that the K562 is transformed.

Cell line	Plate no.	Field number																				% in 10 Fields	
		1		2		3		4		5		6		7		8		9		10			
		C[a]	S[b]	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
K562	1	5	2	5	1	6	4	1	4	2	3	3	1	3	1	3	1	3	1	4	2	64	36
	2	4	3	4	0	3	2	3	2	3	0	2	1	4	0	0	2	2	0	3	4	67	33
3T3	1	0	4	0	4	1	2	0	4	0	2	0	2	0	3	1	4	0	4	0	5	5	95
	2	0	2	0	5	0	2	0	5	0	5	0	3	0	2	0	7	0	2	0	4	0	100
OP6	1	0	2	0	5	0	4	0	2	0	2	0	4	0	2	0	2	0	1	0	5	0	100
	2	0	3	0	4	0	4	0	2	0	3	0	3	0	3	0	2	0	1	0	5	0	100
OP27	1	0	1	0	6	0	3	0	2	0	2	0	5	0	4	0	0	0	2	0	1	0	100
	2	0	2	0	5	0	4	0	3	0	3	0	5	0	4	0	6	0	2	0	2	0	100
OP47	1	0	1	0	3	0	3	0	2	0	3	0	1	0	2	0	3	0	1	0	2	0	100
	2	0	3	0	1	0	2	0	3	0	3	0	2	0	2	0	4	0	3	0	1	0	100
OP55	1	0	1	0	3	0	4	0	5	0	3	0	2	0	1	0	3	0	3	0	2	0	100
	2	0	3	0	2	0	3	0	4	0	2	0	4	0	2	0	2	0	2	0	2	0	100

[a] = The number of colonies i.e. a cluster of cells greater than 3 cells
[b] = The number of single colonies

3.4 Discussion

The well defined lineage of olfactory epithelial cells and the ready availability of reliable markers for stages along the olfactory developmental pathway make this a particularly attractive system to study neurogenesis.

I decided to use murine embryo E10.5 olfactory placodes as the starting material in my attempts to isolate and immortalise precursor cells or stem cells that could give rise to olfactory receptor neurons or GnRH neurons *in vitro*. Such primary cultures tend to grow easily; they are readily disaggregated; they migrate rapidly and they undergo mitosis very soon after explantation, thus providing a good source of material for tissue culture (Paul 1975). At the stage of embryogenesis (E10.5) at which placodes were removed, the different structures of the embryo are clearly distinguishable (Cuschieri & Bannister 1975), and I was thus able to dissect the olfactory placodes without difficulty. Furthermore, E10.5 precedes the stage at which most of the cells leave the cell cycle, and start to differentiate, and when the GnRH neurons begin to migrate out of the olfactory epithelium towards the hypothalamus (Schwanzel-Fukuda & Pfaff 1989; Wray *et al.* 1989a). E10.5 also preceeds differentiation of olfactory receptor cells (E12) and supporting cells (E14) (Sullivan *et al.* 1995; Suzuki & Takeda 1993).

I used C3H mice since this is the inbred strain from which hypogonadal (*hpg*) mice arose spontaneously (Cattanach *et al.* 1977). The defects in the *hpg* mouse are due to a severe truncation of the gene that encode proGnRH so that a portion of the mRNA is transcribed but none is translated (Mason *et al.* 1986). Both male and female *hpg* animals have immature reproductive systems with infantile gonads and are consequently infertile (Cattanach *et al.* 1977). Had I been successful in immortalising GnRH precursor cells, I hoped to transplant them back into *hpg* mice to induce gonadal recovery. Histocompatability between placode cells and intended recipients was thus important.

I isolated sixty possible olfactory placode cell lines after infection of the primary cultures with recombinant transforming retrovirus. Each cell line was initially selected on the basis of resistance to G418. Those that survived were cultivated at 33°C to yield, eventually 48 permanent lines. The other 12 cell lines were lost to crises or contamination.

By using developmental markers to characterise the phenotypes of the 48 cell lines, I reduced the

number to four that, I thought, merited further study. These (OP6, OP27, OP47 and OP55) and the pattern of marker transcripts they expressed are listed in Table 3.1.

All cell lines expressed the Olf-1 mRNA. This was originally identified in adult neurons (Wang & Reed 1993; Wang *et al.* 1993) and considered to be a highly specific olfactory neuronal marker. It was subsequently shown, however, to be identical to the EBF factor (Hagmann *et al.* 1993) and rather widely expressed during embryogenesis.

None of the cell lines, when grown at 33°C expressed the more olfactory-specific marker, Mash-1. Mash-1 is an important transcription factor that is involved in the proliferation and differentiation of progenitor cells in the olfactory epithelium. In its absence olfactory progenitor cells fail to differentiate into mature olfactory receptor neurons. Mash-1^+ cells would, therefore, be ideal precursor cells to immortalise.

The progenitor cells of the olfactory epithelium give rise to two types of neurons: the olfactory receptor neurons and the GnRH neurons.

Olfactory receptor neurons are derived from local stem cells. They remain in the olfactory epithelium where they mature and are responsible for transduction of smell. None of the cell lines expressed a transcript for olfactory receptor expression. This was to be expected since the onset of olfactory receptor expression is observed between E11.5 and E14 (Cuschieri and Bannister 1978; Grazaidei & Monti Grazaidei 1979; Strotmann *et al.* 1995; Sullivan *et al.* 1995) and my primary cultures were generated from E10.5. Furthermore, olfactory receptor transcripts would be expressed by cells that have left the cell cycle and started to differentiate into the mature phenotype. The SV40 retrovirus, however, only integrates into rapidly dividing cells.

The GnRH neuronal progenitor cells become postmitotic from E10-E11 (Schwanzel-Fukuda & Pfaff 1989) when they start to express the GnRH transcript and migrate towards the hypothalamus. Three of the four cell lines chosen, OP6, OP47 and OP55, expressed a very faint transcripts for GnRH. These cell lines may have represented progenitor cells that had started to express the marker but had not yet differentiated into mature GnRH neurons.

Nestin a multipotential stem cell marker in the CNS was expressed by OP6 and OP55 but not by OP27 or OP47. OP6 and OP55 may have represented precursors of olfactory receptor neurons or of multipotential precursor cells that could differentiate into neurons or glial cells.

None of the cell lines expressed the olfactory glial cell marker, $p75^{NGFR}$. $P75^{NGFR}$ is abundantly synthesised in neuronal and non-neuronal tissue of young animals. In adults its expression is more limited, but the presence of $p75^{NGFR}$ was reported in several categories of neurons as well as in other tissues such as muscle, inner ear, testes or in submaxillary glands (Chao 1994). In other sensory systems, $p75^{NGFR}$ has been shown to play an important role in the development and function of sensory neurons (Lee *at al.* 1992; Ugolini *et al.* 1995).

OP27 and OP47 expressed the transcript for Pax6, a transcription factor that has been shown to be expressed in horizontal basal cells and sustentacular cells in the olfactory epithelium. During early embryogenesis, Pax-6 is known to be more broadly distributed, in a similar fashion to Olf-1 expression (Davis & Reed 1996). Later in embryogenesis Pax6 is down-regulated when cells become postmitotic and start to differentiate. Pax-6, a non-neuronal olfactory marker is present in the optic neuronal lineage at different stages of development (Davis & Reed 1996; Grindley *et al.* 1995, Kawakami *et al.* 1997).

To identify proliferating progenitor cells of the olfactory epithelium, primers were designed to the transcription factor BF-1. BF-1 plays an important role in the proliferation and timing of differentiation of olfactory and telencephalon progenitor cells. OP6, OP27 and OP47 all expressed the BF-1 marker, implying that these cell lines were immortalised from the olfactory or the telencephalon population of progenitor cells. The telencephalic origin of the cells could not be excluded as it was possible that I included part of the telencephalon in my primary cultures.

The four cell lines chosen had different phenotypic expression profiles as determined by RT-PCR using markers for different cell types in the olfactory epithelium or progenitor cells. OP6 and OP55 had similar expression patterns in that they expressed the same markers except for BF-1, which was expressed in OP6 but not OP55 (Table 3.2). OP6 and OP55 expressed markers of the olfactory and neuronal phenotype, viz. Olf-1, GnRH and nestin but not the non-neuronal marker Pax-6 nor p75. It is possible that these two cell lines could be precursors of olfactory receptor neurons and could be induced to differentiate along this pathway under the correct *in vitro* conditions.

The other two cell lines, OP27 and OP47 were closer to each other in their expression patterns than they were to OP6 and OP55. Interestingly, OP27 and OP47 differed from OP6 and OP55 in that they lacked expression of nestin, a neuronal marker for chemosensory precursors (Osada *et al.* 1995) but expressed Pax-6, which has been implicated in the non-neuronal olfactory lineage. It is possible that OP27 and OP47 were precursor cells of the non-neuronal lineage or that I isolated a

very early precursor cell or multipotential stem cell that had not yet expressed the nestin marker (Jackson *et al.* 1981).

Pax-6 expression was detected in horizontal basal cells and sustentacular cells in the adult olfactory epithelium (Davis & Reed 1996), suggesting that horizontal basal cells are the precursors of sustentacular cells (Davis & Reed 1996; Huard *et al.* 1998; Suzuki & Takeda 1991b). Other researchers have proposed that horizontal basal cells, or a subpopulation thereof, are stem cells for olfactory receptor neurons (Satoh & Takeuchi 1995; Schwartz Levey *et al.* 1991). This argument has been strengthened by the Mash-1 knockouts where the homozygotes had severely affected olfactory epithelium that lacked olfactory receptor neurons, horizontal basal cells and globose basal cells (Guillemot *et al.*1993). At this point we cannot exclude the possibility that the OP27 and OP47 cell lines, which expressed Pax6 and lacked nestin expression, were either precursors of neuronal or non-neuronal cells.

These results add to those of others who have established immortalised cultures from the olfactory epithelium, either by spontaneous immortalisation or by the transduction of a proto-oncogene, as discussed in Chapter 1. These cell cultures have all been extremely useful, but none have had the advantage of being manipulable between different states of differentiation. By using a retrovirus: SV40 construct, in which the immortalising gene encoded a temperature-sensitive SV40 Tag, I hope to have overcome this problem.

The OP cell lines that I have produced proliferate indefinitely as primitive cells at 33°C. When shifted to 39°C the SV40 Tag is inactivated, proliferation ceases. The four cell lines all showed different population doubling times and saturation densities.

The thermolabile SV40 Tag protein is degraded at 39°C (Tegtmeyer 1975). This was confirmed, in my experience, by the absence of immunoreactivity to SV40 antibody in any of the OP cells maintained for 10 days at 39°C [Fig 3.13 (B, D, F, H)].

Generally speaking, when precursor cells differentiate they do so in a "terminal" manner so that, *pari passu* with progressive expression of the mature phenotype, proliferation becomes limited and eventually ceases, the cells become senescent and death finally ensues. This is the model that applies *in vivo* to all surface epithelia, including the olfactory epithelium. The *in vitro* system that I have developed for studying cellular differentiation accords with this model inasmuch as a shift in temperature to 39°C, by removing the influence of the SV40 Tag and presumably initiating a

program of differentiation, resulted in a fall in the rate of cell division with eventual mitotic arrest.

It is of interest, however, that this arrest was not irreversible: a shift back to 33°C restored the action of the SV40 Tag and proliferation recommenced. The innate and inexorable terminal program that is initiated with differentiation *in vivo* was reversible *in vitro.*

The re-appearance of the immortalised phenotype when the cells were shifted back to the permissive temperature indicates that cellular proliferation was controlled by the SV40 Tag and that the viral DNA was not excised nor lost from the cells when reversion occurred. The cells were only shifted from 33°C → 39°C → 33°C once. In these respects my results confirm those of others who have worked with the temperature sensitive SV40 Tag (Brugge & Butel 1975; Osborn & Weber 1975; Tegtmeyer 1975).

The cessation of growth seen at 39°C was not due to thermal injury or to other trivial cause since 3T3 cells thrived at this temperature - growing, in fact, better than they did at 33°C. Furthermore, 39°C is close to the normal body temperature of the mouse, from which these cells were derived.

Mitotic arrest alone is insufficient to justify the claim that the cells were induced to differentiate. A more convincing reason to believe that this did occur is provided by the striking morphological and other changes that I present and discuss in subsequent chapters.

Working with different cell types, other researchers have obtained essentially similar results to those that I obtained.

Jat and Sharp (1989) used the pZipSV*tsA58* retrovirus to establish clonal cell lines from rat embryo fibroblasts (REF). They selected for transformed cell lines by growing in the presence of the neomycin analogue, G418. They found that the SV40 Tag mutant immortalised REF cells at the permissive temperature of 33°C; when shifted to the non-permissive temperature of 39°C, growth was abolished. The cells stopped dividing within 24 hours of transfer to 39°C and they arrested in either the G_1 or the G_2 phase of the cell cycle, proving that the SV40 Tag was required for initiation and maintenance of the immortal phenotype and that the SV40 Tag was required for the cells to proceed through these stages of the cell cycle. But the cells remained metabolically active (as assayed by general protein synthesis) for 6 days after growth arrest. When the temperature sensitive cells were shifted from 39°C back to 33°C after 48-72 hours, none of the cells re-entered the cell cycle indicating that, unlike the situation that obtained with my cells, mitotic arrest was irreversible.

Precursor cells from the CNS (Fredriksen *et al.* 1988), E17 Hippocampal cells (Mehler *et al.* 1993) and Raphe temperature sensitive cell lines (Stringer *et al.* 1994) were isolated by immortalisation with the SV40 tsA58 mutant retroviral vector. These precursor cells all showed evidence of being multipotential when shifted to the non-permissive temperature and in the presence of different growth conditions or different growth factors that could induce the cell line to follow one path or another. Stringer *et al.* (1994) showed that the Raphe precursor cells followed the neuronal pathway in the presence of basal medium but chose the astrocyte pathway in the presence of high serum. These cell lines were not able to be maintained at 39°C, showing the importance of SV40 Tag for maintenance of the immortalisation phenotype.

Almazan and McKay (1992) constructed a double mutant retrovirus vector containing both the *A58* and the U19 mutation of the SV40 Tag. This hybrid was constructed to increase the probability of immortalisation (Jat & Sharp 1986). They used this construct to immortalise a neuronal precursor cell from rat optic nerve. The clonal cell line (tsU19-5) displayed some properties of oligodendrocyte precursors in that it proliferated, bound the monoclonal antibody A2B5 (which recognises minor ganglioside species), expressed the intermediate filament vimentin and the enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) at 33°C. At 39°C, some cells differentiated further and expressed several oligodendrocyte specific components. These results showed that conditional oncogenes can establish neural precursor cell lines that are still capable of differentiation *in vitro*.

The SV40 Tag has the ability to transform or immortalise cells that express it. This is an important distinction, since these cell lines I derived were developed for ultimate transplantation work. Transformed cells will form tumours and would not be useful in transplantation work. Using the soft agar assay, I showed that the OP cell lines were unable to grow in soft agar, unlike the malignant K562 cell line, and could thus be regarded as immortalised rather than transformed.

Chapter 4

The OP cell lines differentiated into olfactory receptor expressing neurons upon shifting to the non-permissive temperature.

4.1 Summary

Four immortalised cell lines from the olfactory epithelium have been characterised at the permissive temperature of 33°C. At this temperature the four OP cell lines differed morphologically. When these OP cells were shifted from 33°C to 39°C, the SV40 Tag was inactivated, continuous proliferation ceased and differentiation commenced. The OP cell lines were induced to differentiate by a shift in temperature or by the addition of the morphogen, retinoic acid. The induction resulted in the four cell lines sharing some common features but they also showed distinctive characteristics.

All four cell lines expressed, β-actin and Olf-1 under all growth conditions and none expressed GnRH, vimentin, NF160, GFAP or O1. The expression of the other markers differed for the different cell lines.

The induction of differentiation by a shift to 39°C and / or RA resulted in the expression of olfactory receptor genes which could be detected in all four cell lines by RT-PCR with degenerate primers.

A shift to 39°C in DM-10 resulted in two morphological types becoming prominent. These cell types were not detected at 33°C. In the case of OP6, OP27 and the OP47 cells, a large, flat cell type and refractile cells with unipolar or multipolar extensions appeared. Two different cells - a large and a small cell type - were observed in the OP55 cell line. The small cells often formed closely packed groups of cells around the large cell type. A shift to 39°C in RA did not have the same effect on morphology, except for the OP47 cells. The cells in RA medium were more uniform.

The OP6 cells differentiated along a neuronal pathway, developed neurite-like extensions and started to expressed BF-1 and OR transcripts. Expression of the stem cell marker, nestin, was down-regulated when shifted to 39°C. They did not express Mash-1 and Pax6 which were induced in the other cell lines.

The OP27 cells differentiated from a keratin$^+$/nestin$^+$ population at 33°C characteristic of horizontal basal cells to become olfactory receptor-expressing neurons that continued to express Olf-1, nestin, keratin, BF-1, Otx2 and started to express Pax6 and OR transcripts when shifted to 39°C. OP27 cells were negative for Mash-1 at 33°C and 39°C.

The OP47 cell line comprised early stem cells which continued to express Olf-1 and only started to express nestin once shifted to 39°C. This increase in expression of nestin was accompanied by an increase in expression of Mash-1, olfactory receptor transcripts, BF-1 and the down regulation of the Otx2 marker. The OP47 cells were not induced to express either the Pax6 or the keratin markers.

When shifted to 39°C, OP55 cells differentiated along the neuronal pathway, with expression of Mash-1, BF-1 and olfactory receptor transcripts. Otx2 was continuously expressed and nestin expression was down regulated. Keratin immunoreactivity was detected at 33°C but a shift to 39°C in DM-10 resulted in the loss of keratin while a shift to 39°C in RA saw the continued expression of keratin. OP55 cells also remained negative for the Pax6 marker.

4.2 Introduction

The essential goal of this project was to develop cell lines of olfactory placode origin that could be used to study differentiation *in vitro* and *in vivo*. This required:

a) that embryonic placode cells be immortalised at a relatively primitive stage of their development

b) that they proliferate continuously while maintaining their undifferentiated phenotype and finally,

c) that their differentiation to mature cells be inducible.

As recorded in the previous chapter, the first two of these requirements have been met.

Four cell lines (OP6, OP27, OP47 and OP55) were established from the olfactory placode of E10.5 mice embryos. This was achieved by infection with a retrovirus carrying a temperature sensitive allele of the SV40 Tag.

At the permissive temperature of 33°C, the four cell lines proliferated and expressed the SV40 Tag protein continuously. A shift to the non-permissive temperature resulted in the loss of SV40 Tag expression and the cells stopped dividing. Clonality of the cell lines was confirmed by Southern blot analysis.

In this chapter I present the results of experiments designed to address the third of the above requirements - i.e. that the cells could, indeed, be induced to differentiate. These experiments involved exposing the cells to a temperature of 39°C, to retinoic acid, or to a combination of the two.

The rationale behind the temperature shift has been discussed in detail in previous chapters. Since immortalisation and, presumably, the primitive state was maintained by a temperature sensitive SV40 Tag, incubation of the cells at the non-permissive temperature should have arrested continuous proliferation and allowed maturation to proceed.

The use of retinoic acid was based upon an extensive literature attesting to the morphogenic properties of this molecule.

4.2.1 *Retinoic acid in development*

Retinoic acid (RA), a natural metabolite of vitamin A (retinol), plays a key role in vertebrate development. It binds to nuclear retinoic acid receptors (RARs) and so functions as an endogenous signalling molecule (Brand *et al.* 1988; Giguere *et al.* 1987; Petkovich *et al.* 1987).

RA is present in *Xenopus* embryos at a concentration of 1.5x 10^{-7} M (Durston *et al.* 1989) and at 5x 10^{-8} M in the chick limb bud (Thaller & Eichele 1987). Its endogenous presence has thus been confirmed. It is also known that the intracellular concentration of retinoids is regulated by the binding proteins cellular retinoic acid binding protein (CRABP) and cellular retinol binding protein (CRBP). The function of CRBP is to store and release retinol in situations where high concentrations of RA are required; CRABP serves to sequester RA in cells when low concentrations of RA are called for and interaction of RA with its nuclear receptors is to be prevented (Dolle *et al.* 1990).

RARs bind to all-trans RA with high affinity. These receptors belong to the steroid/thyroid hormone family of nuclear receptors that act as transcription factors. Three different mammalian receptors have been identified; RAR α (Giguere *et al.* 1987; Petkovich *et al.* 1987), RAR β (Benbrook *et al.* 1988; Brand *et al.* 1988) and the RAR γ (Krust *et al.* 1989; Zelent *et al.* 1989). RAR α transcripts appear ubiquitously distributed in the mouse fetus, while RAR β and RAR γ transcripts are more restricted in their pattern of expression (Dolle *et al.* 1990).

After binding to the nuclear receptor(s), the RA-RAR complex regulates gene expression by interacting with retinoic acid response elements (RAREs) present in the promoters of responsive genes (Langston & Gudas 1992; Love & Gudas 1994; Mangelsdorp *et al.* 1991; Nagpal *et al.* 1992). RA can act both as a positive and negative regulator of gene transcription. It up-regulates, for example, expression of the homeobox-containing *Hox* genes (Simeone *et al.* 1990; 1992) and down-regulates the expression of the Otx2 gene (Simeone *et al.* 1993; 1995).

A number of *in vitro* and *in vivo* studies have attested to the effects of RA on differentiation and embryogenesis (Avantaggiato *et al.* 1996; Durston *et al.* 1989; Holder & Hill 1991; Husman *et al.* 1989; Jones-Villeneuve *et al.* 1982; Kessel & Gruss 1991; Papalopulu *et al.* 1991; Simeone *et al.* 1990; Simeone *et al.* 1995).

When added to the embryonal carcinoma cell line P19, RA caused the cells to differentiate into neurons that expressed NCAM and neurofilament markers and into glial cells that expressed GFAP (Husman *et al.* 1989). Since the P19 cell line is innately committed to developing into neurons, glial cells or fibroblasts, it is probable that RA induces differentiation without determining a specific cell type (Jones-Villeneuve *et al.* 1982). In other studies Simeone *et al.* (1990) showed that the expression of homeobox-containing *Hox* genes in the human embryonal carcinoma cell line NT2/D1was regulated by retiniods in a time- and dose- dependent fashion.

The responses of *Hox* genes to RA depend upon the presence of the downstream RAREs identified by Langston & Gudas (1992, 1994) and are related to their position of the genes in the chromosome with 3` genes being activated first and to the greatest degree and the most 5` genes being unresponsive (Morriss-Kay 1992).

RA-induced alterations in the establishment of the anterior-posterior identities in the CNS are accompanied by re-patterning of homeobox-containing *Hox* gene expression domains in the hindbrain and the spinal cord (Conlon & Rossant 1992; Marshall *et al.* 1992). Excess RA has a posteriorizing effect. The severity of this effect depends on the dosage and the time of administration to the developing embryos (Durston *et al.* 1989; Papalopulu *et al.* 1991). *Xenopus* embryos treated with RA failed to develop a forebrain, nasal pits or eyes whereas the hindbrain and the spinal cord was increased and the *Hox* gene mRNA, which is only expressed posteriorly in the *Xenopus* larva, showed substantially increased expression. There was no reduction in the volume of the CNS, suggesting that RA did not prevent the formation of the neural structures but instead influenced the way in which they developed (Durston *et al.* 1989).

The effect of RA was shown to be stage dependent with the most dramatic effect on the mouse embryo being at E7.2-7.4, when embryonic cells have acquired the ability to respond to RA but endogenous RA synthesis has not yet started (Simeone *et al.* 1995). Applied at this time the exogenously administered compound resulted in the loss of the forebrain structures and forebrain markers (e.g. Otx2, *Emx1, Emx2* and *Dlx1*) while at the same time, and in the same cells, the expression of the hindbrain markers (e.g. the *Hox* genes, *Pax2, Wnt1, Wnt2, En1* and *En2*) was stimulated, with re-patterning of the forebrain, midbrain and the hindbrain (Avantaggiato *et al.* 1996; Simeone *et al.* 1995).

More specifically, the involvement of RA in development of the olfactory system is apparent from a number of observations.

Firstly, as mentioned above, treatment of *Xenopus* embryos with RA results in the loss of the forebrain structure and the nasal pits (Durston *et al.* 1989).
Secondly, Dolle *et al.* (1990) have shown that RAR and CRABP are expressed in the olfactory epithelium of mouse embryos at E14.5 (Dolle *et al.* 1990). CRABP expression was detected in the developing olfactory receptor neurons but not in stem cells while RAR-β was detected in olfactory supporting cells and RAR-α was found to be expressed in all the cell types.

Thirdly, during early embryogenesis the mesoderm which forms between the olfactory pits and the forebrain is a rich source of retinoids that are oxidised to RA which, in turn, binds to local RARs and results in the expression of a number of genes (Conlon & Rossant 1992; Marshall *et al.* 1992; Morriss-Kay *et al.* 1991). Developing neurons in the olfactory epithelium start to differentiate and their axons grow into the forebrain to make contact with the olfactory bulb - the target organ of the olfactory receptor neurons. In the mouse, RA continues to be produced by the frontonasal mesenchyme once initial morphogenesis of the olfactory pathway is completed (LaMantia *et al.* 1993).

Fourthly, Anchan *et al.* (1997) showed that RA is present in the developing forebrain where it influences the olfactory pathway during initial morphogenesis. These authors evaluated the relationship between abnormal olfactory pathway development and local retinoid signalling in homozygous $Pax6^{Sey\text{-}Neu}$ /$Pax6^{Sey\text{-}Neu}$ mice. In these mutant mice the olfactory epithelium and the olfactory bulb failed to develop and this loss of a primary olfactory pathway is accompanied by the absence of retinoid-producing cells in the frontonasal mesenchyme and the absence of retinoid-mediated gene expression in the olfactory placode and ventrolateral forebrain. These results strongly suggest that retinoid-mediated induction is an essential step in the initial morphogenesis of the mammalian olfactory pathway.

Finally, RA is produced in the frontonasal mesenchyme between E11.5-E13.5 of the mouse embryo with concomitant activation of gene expression (increase in RARβ, CRABPI and CRABPII genes) in a bilaterally symmetrical subset of olfactory receptor neurons in the dorsolateral olfactory epithelium. These elegant observations were made in transgenic mice carrying the gene for β-galactosidase under the control of the promoter for thymidine kinase (a RA response element) gene expression. Expression of this gene is known to be induced by retinoids. Axons from these olfactory receptor neurons extend through the frontonasal mesenchyme toward the forebrain. *In vitro*, RA potentiates the growth of olfactory receptor neuronal neurites on laminin. In the embryo laminin is found in a stripe of frontonasal mesenchyme directly associated with the olfactory nerve. These results, therefore, suggest that RA influences the growth and adhesive properties of olfactory receptor neuron axons (Whitesides *et al.* 1998).

4.2.2 *Markers used to identify the differentiation of the OP cell lines*

In this study, cellular differentiation was measured morphologically and by the expression of genes whose transcription and translation is characteristic of the olfactory epithelial phenotype. Reverse transcription followed by PCR amplification (RT-PCR) was used to detect mRNA transcripts of

selected genes or immunocytochemistry (ICC) with specific antibodies was employed to detect the final translated products of these genes.

Several of these genes [Olf-1, nestin, Mash-1, BF-1, Pax6, GnRH and olfactory receptor (OR) transcripts] and their transcriptional regulation have already been discussed in Chapter 3 (Section 3.2.2). In the experiments described in this chapter, I added the additional markers: keratin, vimentin, glial fibrillary acidic protein (GFAP), neurofilament 160 (NF160), O1 and Otx2.

Keratin, nestin, vimentin, NF160, GFAP are intermediate filament proteins which characterise different cell types in the olfactory neuroepithelium.

Intermediate filaments can be classified into six classes based on expression patterns, sequence similarities and the positions of the introns in their genes.

- Class I and Class II intermediate filaments are, respectively, the basic and acidic keratins that are expressed in epithelia.
- Class III includes desmins, GFAP, peripherin and vimentin.
- Class IV contain the three neurofilament subunits (light, medium and heavy: NF-L, NF-M, NF-H) and α-internexin.
- Class V contains the nuclear lamins.
- Class VI contains nestin (Dahlstrand *et al.* 1992; Lendahl *et al.* 1990; Xu *et al.* 1994).

Keratin belongs to the class of intermediate filaments characteristic of epithelial cells (Moll *et al.* 1982). In olfactory epithelium keratin is found specifically in horizontal basal cells but not in globose basal cells, olfactory neurons or supporting cells. A specific antibody (broad spectrum polyclonal antiserum directed against cytokeratins) labels the horizontal basal cell population (Calof & Chikaraishi 1989; Suzuki & Takeda 1993).

Nestin gene expression serves as a marker for pluripotential stem cells in the CNS and distinguishes them from their more differentiated progeny, the neurons and the glial cells. Low levels are also found in skeletal muscle precursors (Hockfield & McKay 1985, Zimmerman *et al.* 1994). From postnatal day 1-8, the olfactory vomeronasal epithelium consists mainly (80%) of nestin positive cells. From postnatal day 8-22, nestin expression decreases and eventually becomes restricted to the basal layer of the vomeronasal epithelium. These findings led Osada *et al.* (1995) to conclude that nestin expression identifies vomeronasal chemosensory precursor cells in adult mice.

Vimentin is an intermediate filament protein that is found in virtually all proliferating neuroepithelial and neural crest cells, including neuronal and glial precursors. Although, in the CNS generally, there is a brief period during which vimentin and neurofilament proteins are co-expressed in immature neurons and their precursors, postmitotic neurons usually stop expressing vimentin and neurofilament proteins accumulate. Olfactory receptor neurons deviate from this general pattern in that they continue to express vimentin and fewer than 0.1% express neurofilament proteins in the adult (Schwob *et al.* 1986). Thus the olfactory epithelium retains juvenile features inasmuch as the neurons continue to express a protein characteristic of neuronal precursors and young neurons elsewhere. Vimentin is expressed in mesenchymal cells and transiently in neuronal, glial and muscle precursor (Osada *et al.* 1995). Researchers studying the olfactory epithelium have used vimentin as a marker for olfactory neuronal precursors (MacDonald *et al.* 1996), ensheathing cells (Goodman *et al.* 1993) and olfactory neurons (Calof & Guevara 1993; Chuah *et al.* 1991; Goldstein *et al.* 1997; MacDonald *et al.* 1996; Mahanthappa & Schwarting 1993; Schwob *et al.* 1986).

GFAP is an intermediate filament protein found in the CNS and the PNS. It is expressed in astrocytes, glial cells, Schwann cells (Jessen & Mirsky 1980) and in oligodendroglia or immature oligodendrocytes (Choi & Kim 1984). In the olfactory epithelium GFAP is found in olfactory ensheathing cells (Goodman *et al.* 1993) and in non-myelinating Schwann cells, both *in vivo* and *in vitro* (Calof & Guevara 1993). Unlike myelinating Schwann cells elsewhere in the PNS, olfactory ensheathing cells or Schwann cells support, but do not myelinate, the axons of the olfactory receptor neurons (Farbman 1992).

The monoclonal anti-glycolipid O1 antibody is a stage specific-marker for developing oligodendrocytes and is expressed after the progenitor marker, O4, in the CNS (Sommer & Schachner 1981). It is expressed in association with galactocerebroside (GalC) - the major glycolipid component of myelin and the cell-surface component of cultured murine cerebellar cells. The O1 antigen is recognised by a monoclonal antibody that was generated by immunisation of mice with bovine corpus callosum.

The O1 antibody does not react with the surfaces of astrocytes, neurons, fibroblasts or Schwann cells in the CNS or the PNS (Schachner *et al.* 1981; Sommer & Schachner 1981). In culture O1-positive cells have a "hairy-eye ball" morphology, i.e. a cell with abundant bushy processes (Schachner *et al.* 1981). The main functions of oligodendrocytes are to form myelin and to provide nutrition for neurons (Sommer and Schachner 1981).

NF160 is a class IV neurofilament whose expression appears soon after terminal neuronal differentiation. It has been suggested (Xu *et al.* 1994) that neurofilaments determine the radial size of axons by becoming extensively phosphorylated by kinases. The large number of negative-charged phosphates added to the tail domain of the neurofilaments and the few to the head end results in an increase in electrostatic repulsion between the molecules causing wider interfilament spacing and thus axonal expansion.

OTX2 is a vertebrate homeobox gene related to the *orthodendricle*, a Drosophila gene implicated in the control of head development in the fruit-fly. Otx2, originally isolated in the mouse (Simeone *et al.* 1992), is transcribed in the embryonic ectoderm or epiblast as early as E5.5-5.7 post-coitum (Simeone *et al.* 1993). Between E7 and E7.5, Otx2 expression progressively recedes to the anterior regions during gastrulation where it remains confined to the neuroectoderm of the headfold (Simeone *et al.* 1993). This restriction of Otx2 expression to the diencephalon, mesencephalon, telencephalon and the forebrain suggests a role for the Otx2 in the development of the head.

This suggestion is supported by the observation (Pannesse *et al.* 1995) that over-expression of Otx2, achieved by microinjection of increasing amounts of synthetic Otx2 mRNA into *Xenopus* zygotes, resulted in increasingly shortened embryos with severely truncated trunks and tails and an expansion of the head structures.

Furthermore, homozygous null Otx2 mutants caused by gene disruption experiments in mice, produced in two different labs (Acampora *et al.* 1995; Ang *et al.* 1996), deletion of the rostral brain regions anterior to the rhombomere 3, including the forebrain and the midbrain. In addition, these mutants failed to gastrulate properly and stopped development at early midgestation.

Otx2 is widely expressed early in embryogenesis in precursor cells in the anterior regions of the developing nervous system, including the olfactory epithelium, at a time when neurons are actively generated (Simeone *et al.* 1993). Otx2 has been shown to be expressed by a significant proportion (42%) of migratory cells in olfactory explant cultures that consist mainly of olfactory neurons and their precursors, the INPs (Calof *et al.* 1996). Since Otx2 expressing cells lack neurites, these authors suggested that Otx2 represents a global marker for the olfactory receptor neuronal progenitor cells. The expression of Otx2 in migrating cells has been confirmed by the studies of Mallamaci *et al.* (1996) who showed expression of Otx2 in migrating GnRH expressing cells and ensheathing glial cells as they migrated towards the telencephalon. Unlike Calof *et al.* (1996), they showed expression of Otx2 in postmitotic ciliated sensory neurons but they were unable to detect

expression in the basal cells and in the immature olfactory receptor neurons (Mallamaci *et al.* 1996). This discrepancy could be due to the fact that Calof *et al.* (1996) used explant cultures while Mallamaci *et al.* (1996) used mice embryos in their study.

4.3 Results

4.3.1 Protocols for studying differentiation in vitro

OP cells were plated at 33°C in DM-10 and allowed to settle overnight. The medium was then replaced with fresh DM-10 or with N2 medium containing 10^{-6} M RA. When 60-70% confluent, test cultures were shifted to 39°C where they were maintained, with medium changes every 3-4 days, for assessment after 4, 8, 12 and 18 days. Cultures were examined microscopically for morphological evidence of differentiation and by RT-PCR and ICC for evidence of induced expression of differentiation markers. Details of the techniques used are presented in Chapter 2.

Controls for the RT-PCR experiments included the use of GT1-7 cell line as a positive control for Olf-1 and GnRH (Illing and Hapgood unpublished data) and as a negative control for the other RT-PCR markers. Appropriate cDNA or gDNA (where cDNA was not available) was used as positive controls for each respective primer pair (Table 2.4) and the water control served as a negative control in the absence of DNA.

As an internal control β-actin primers were used to ensure relatively equal concentrations of cDNA for each reaction. Amplification with the β-actin primers resulted in a relatively equal size band (of 354 bp for cDNA) in all the samples except for OP6 at 39°C (day 18) in the DM-10 medium, the COS-1 cells and the water control [Fig 4.4 (A)]. From these results we see that this OP6 sample and the COS-1 sample lacked β-actin transcripts and were thus used as negative controls to ensure the absence of contaminating products.

To confirm that none of the RT-PCR results were due to contaminating gDNA, the RNA was DNase treated before reverse transcription and the following primer pairs (β-actin, GnRH and Mash-1) were designed across introns to amplify a larger gDNA PCR product than for the cDNA [Figs 3.2 (A); 3.5 (A); 3.10 (A)]. None of the samples amplified a fragment of gDNA for these primers, confirming the absence of contaminating gDNA in the samples [Figs 4.4 (A, F; C), respectively]. The RT-PCR results are presented in Figs 4.4 and summarised in Tables 4.1; 4.3; 4.5 and 4.7. Photographs showing the morphology of the cell lines under different growth conditions are presented in Figs 4.2; 4.3; 4.7; 4.8; 4.10; 4.11; 4.13 and 4.14.

Cells for ICC were fixed and processed at 33°C and at 39°C as described in Fig 2.1. ICC was performed at the dilutions of primary and secondary antibodies described in Table 2.2, using

primary cultures which also served as positive controls for these antibodies [Fig 4.1 (A-F)]. The negative controls were cells processed in the absence of primary antibody. Some cells that were maintained for long periods at 39°C i.e. for 18 days had high background staining which was weaker that the positive controls but stronger than the negative controls. It has been documented that cells that are dying or damaged stain falsely positive during ICC (Bourne, Dako handbook). ICC results are shown in Figs 4.5; 4.9; 4.12 and 4.15 and summarised in Tables 4.2; 4.4; 4.6 and 4.8.

To determine the percentage of positive cells to negative cells, six randomly chosen fields (under 10x magnification) for each time point were chosen and the positive cells counted as a percentage of the total number of cells. Slides with uniform staining were scored as very strongly positive (+++), weaker positive (++), background staining (bg) and no staining (-).

For each of the four cell lines, this protocol thus generated data, on the effects
a) of RA alone (DM-10 cells vs. RA cells at 33°C)
b) of a temperature shift alone (DM-10 cells at 33°C vs. the same cells at 39°C)
c) of the combinations of RA and a temperature shift.

4.3.2 *The OP6 cell line*

4.3.2 (a) OP6 in DM-10 at 33°C vs. RA at 33°C

RA had no discernible effect on the morphology of OP6 cells maintained at 33°C. In both DM-10 and RA medium the cells grew as even monolayers with irregular outlines and large distinctive nuclei. No processes that could be identified as neurites were observed [Figs 4.2 (A) and 4.3 (A)].

At the permissive temperature RA had no effect on the transcription or expression of Mash-1, Pax6, GnRH, Otx2, vimentin, NF160, GFAP or O1 (Tables 4.1; 4.2) nor was the transcription of Olf-1 (seen in DM-10) appreciably increased in RA medium (Table 4.1).

Transcripts of BF-1 [Fig 4.4 (D)] and OR [Fig 4.4 (G)] were induced by RA (Table 4.1).

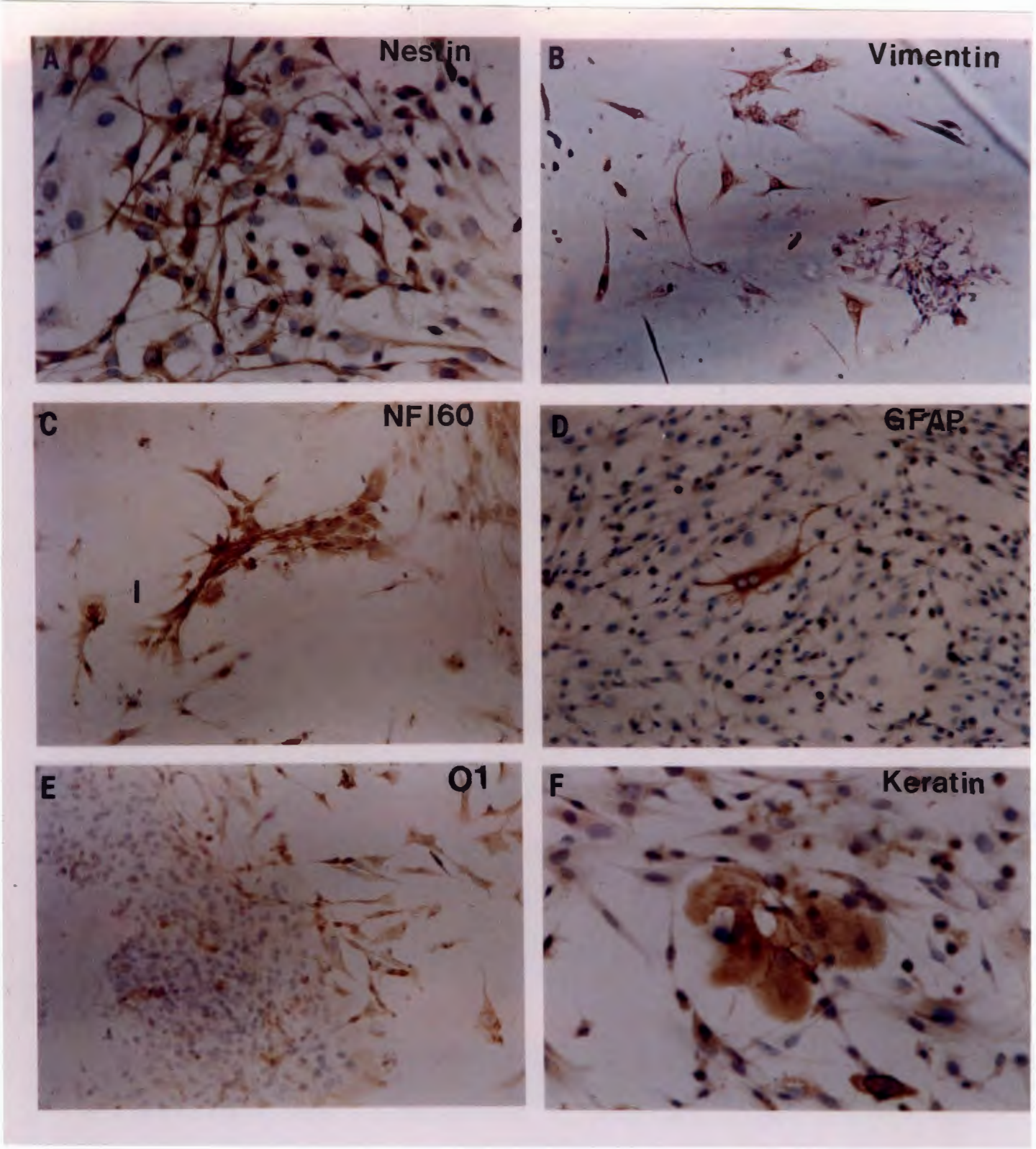


Fig 4.1 Primary cultures used as positive controls for ICC with antibodies to nestin (A); vimentin (B); NF160 (C); GFAP (D); O1 (E) and keratin (F). Cells were visualised with DAB (brown) and counterstained with hematoxylin (blue nuclei). Mag. 100x (A-C, E, F) and 50x (D).

The OP6 cells expressed the nestin protein strongly in DM-10 [Fig 4.5 (A)] and in RA [Fig 4.5 (C)]. Keratin protein that was weakly expressed (4% of cells) in DM-10 was less detectable in RA medium (Table 4.2).

4.3.2 (b) OP6 in DM-10 at 39°C

A shift from 33°C to 39°C in DM-10 medium caused significant changes in the morphology of OP6 (Fig 4.2). Two distinct forms were discernible. The first was characterised by a predominant population of flat, epithelial-like cells (∧) that were larger than those maintained at 33°C and where the ratio of cytoplasm to nuclei was much greater. The second population comprised elongated cells, some of which had long extensions that made contact with the neighbouring large, flat cells. The nuclei of these cells were asymmetrically situated at the ends of the elongated cells and very little cell cytoplasm could be seen [▲, Fig 4.2 (B)].

After 8 days in culture [Fig 4.2 (C)] the two morphologies were still present with the predominating cell type being the large, flat cells (∧). After 12 days in culture at 39°C [Fig 4.2 (D)], the OP6 cells (and in particular, the large, flat cells) started to look unhealthy. The cytoplasmic membranes of the larger cell types had a 'shredded' appearance and numerous small, phase-dense bodies surrounded the cell nuclei. The second cell type, now fewer in number, resembled unipolar or bipolar cells that connected with each other to form a reticulum across the larger cells (▲).

After 18 days at 39°C [Fig 4.2 (E)], few, if any viable cells could be seen. All that remained of the culture was a residual web of cellular remnants composed of fine, irregular filaments between refractile nuclear forms. Floating dead cells or debris were conspicuous by their absence.

The transcription of Mash-1, Pax6 and GnRH were unaffected by growing the OP6 cells at 39°C (Table 4.1). The transcription of Olf-1 continued to be expressed [Fig 4.4 (B)]. Otx2 [Fig 4.4 (H)] transcripts, absent at 33°C, were faintly detectable on day 4 but not thereafter. The transcripts for both BF-1 [Fig 4.4 (D)] and the OR [Fig 4.4 (G)] were negative at 33°C but increased with time once OP6 cells were shifted to 39°C and their expression was maintained for 12 days (Table 4.1). No expression was detected at day 18, when the cells was no longer viable, confirming that the other RT-PCR results were not a result of contamination.

The expression of vimentin, NF160, GFAP and O1 protein, all negative at 33°C in DM-10, remained so at 39°C. I noticed a slight increase in background staining with time at 39°C that was marginally stronger than the negative control but much weaker than the positive control primary culture (Table 4.2).

The OP6 cells expressed nestin protein strongly at 33°C in DM-10 [Fig 4.5 (A)]. The shift to 39°C resulted in a decrease in expression so that only 35% of cells were positive after 4 days with further loss thereafter (Table 4.2). Such OP6 cells that remained strongly nestin positive at 39°C were cells that have started to grow multipolar extensions [Fig 4.5 (B)].

4.3.2 (c) OP6 in RA at 39°C

The morphology of the OP6 cells grown in RA medium at 39°C differed from that seen when the cells were grown in DM-10 at the elevated temperature. Within hours of shifting to 39°C approximately 50% of the cells died leaving small phase-bright cells and thin cells with extensions [Fig 4.3 (B)]. A few unipolar or multipolar cells extended processes which grew longer with the passage of time [▲, Fig 4.3 (E)]. The majority of cells were irregular in shape and lacked extensions (⇑).

Cells that had been maintained for 12 days in culture at 39°C [Fig 4.3 (D)], were reduced in number to about 20% confluency. Many of the cells had an unhealthy appearance and by 18 days in culture [Fig 4.3 (E)], they had become small irregular shaped and phase bright (⇑). The 'ghostly' network seen in DM-10 after 18 days [Fig 4.2 (E)] was not formed when RA was present nor were the two distinct cell types (i.e. one a population of large, flat epithelial cells and the other a population of phase-bright cells with long processes that contacted other cells in culture. In RA there were fewer but more viable cells for a longer period of time compared to cells in DM-10.

As was the case with a shift to 39°C in DM-10 alone, the addition of RA did not result in a change in the transcription of Mash-1, Pax6, GnRH or BF-1 (Table 4.1). Olf-1 expression at 33°C, at 39°C in RA and at 39°C in DM-10 was similar except that transcripts were present at day 18 [Fig 4.4 (B)]. OR transcripts were present in the RA medium at 33°C and 39°C although faintly so at both temperatures. In DM-10 on the other hand OR transcripts were not detected at 33°C and gradually accumulated with time at 39°C [Fig 4.4 (G)].

BF-1 expression at 39°C after 8 days in RA medium was undetectable; at all other time points transcripts were seen [Fig 4.4 (D)] as this does fit the trend of the time course, it may be due to an artefact that arose during the experimental procedure.

The Otx2 transcript was not expressed at 33°C in either DM-10 or RA medium. A shift to 39°C in DM-10 resulted in a faint transient increase in expression at day 4. In contrast expression of Otx2 at 39°C in RA medium, increased steadily to a maximum after 12 days in culture [Fig 4.4 (H)].

OP6 cells were negative at 33°C for vimentin, NF160, GFAP, O1 and keratin protein but a shift to 39°C in RA medium resulted in an increase in background staining especially for the antibodies, NF160 and keratin. The staining in the RA medium was stronger than that seen at 39°C in the DM-10 medium but not as strong as the positive control primary culture (Table 4.2).

Nestin protein was expressed in RA medium at 33°C. A shift to 39°C resulted in a gradual decrease (86% after 4 days) in protein expression [Fig 4.5 (D)]. The nestin gene remained relatively constant thereafter [Fig 4.6 (A); Table 4.2]. In the RA medium the majority of the cells continued to express nestin (82-87%) whereas in the DM-10 medium most of the cells lost nestin immunoreactivity and by 12 days at 39°C only 14% of the OP6 cells were positive [Fig 4.6 (A); Table 4.2].

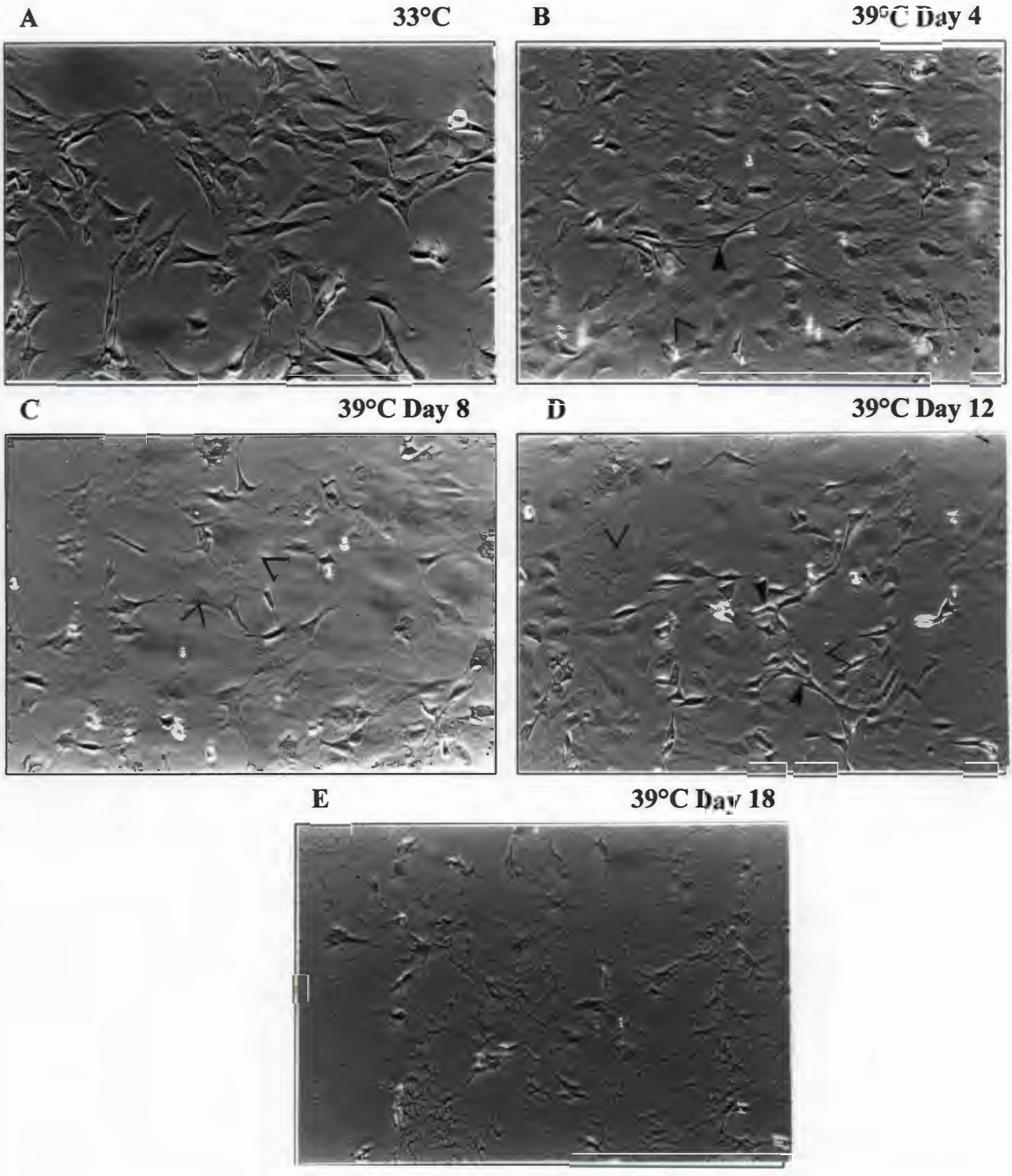


Fig 4.2 Morphology of OP6 cells maintained at 33°C or at 39°C in DM-10 medium. Phase contrast micrographs of OP6 cells at 33°C (A) or 39°C day 4 (B), day 8 (C), day 12 (D), day 18 (E). Cultures were photographed with an inverted microscope at 50x magnification. ∧ represents large, flat, epithelial-like cells and ▲ represents cells with extensions. The morphology is discussed in the results section.

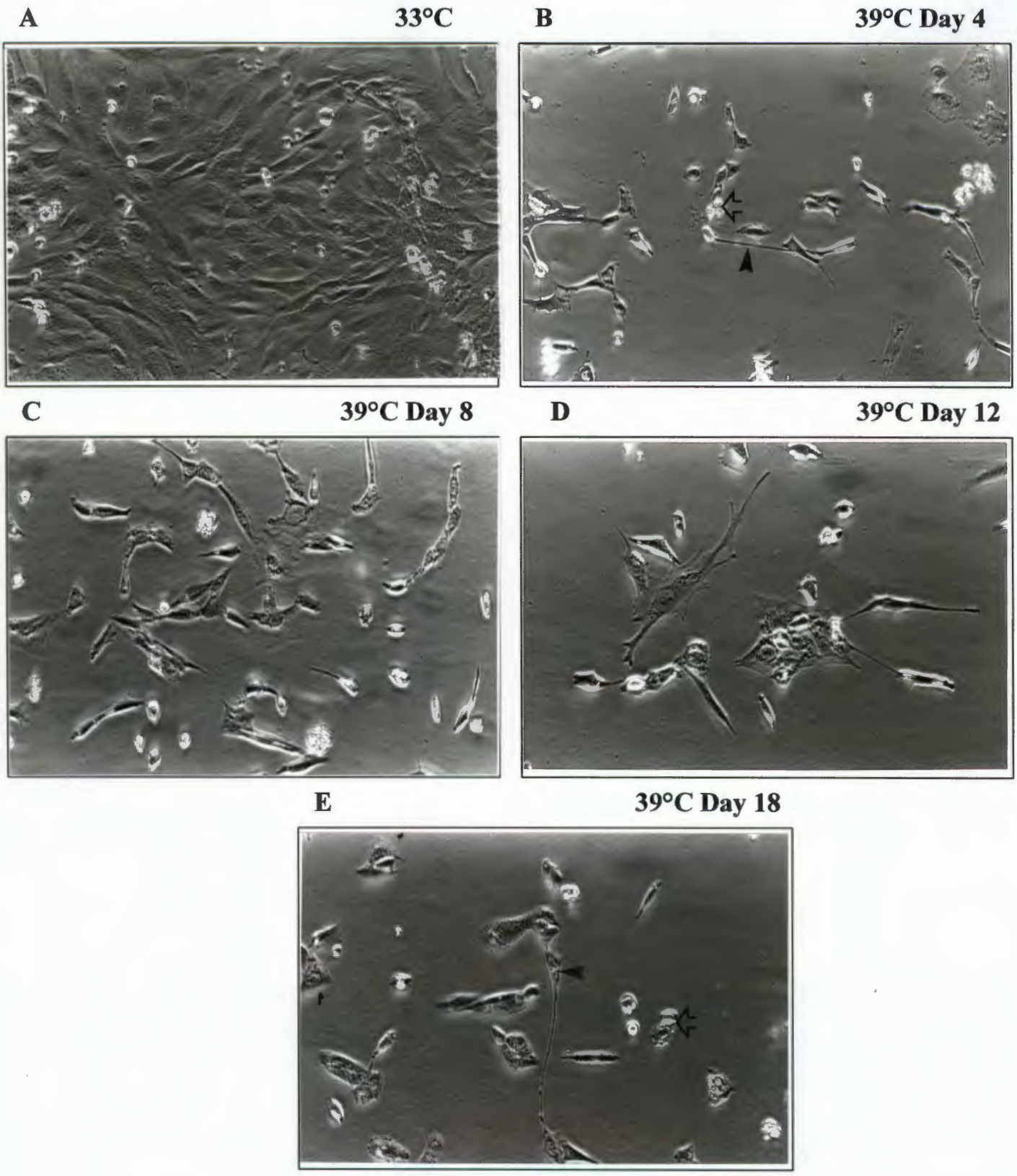


Fig 4.3 Morphology of OP6 cell line maintained at 33°C or at 39°C in RA medium. Phase contrast micrographs of OP6 cells at 33°C (A) or 39°C day 4 (B), day 8 (C), day 12 (D), day 18 (E). Cultures were photographed with an inverted microscope at 50x magnification. ⇧ represents refractile cells without extensions and ▲ represents cells with extensions. The morphology is discussed in the results section.

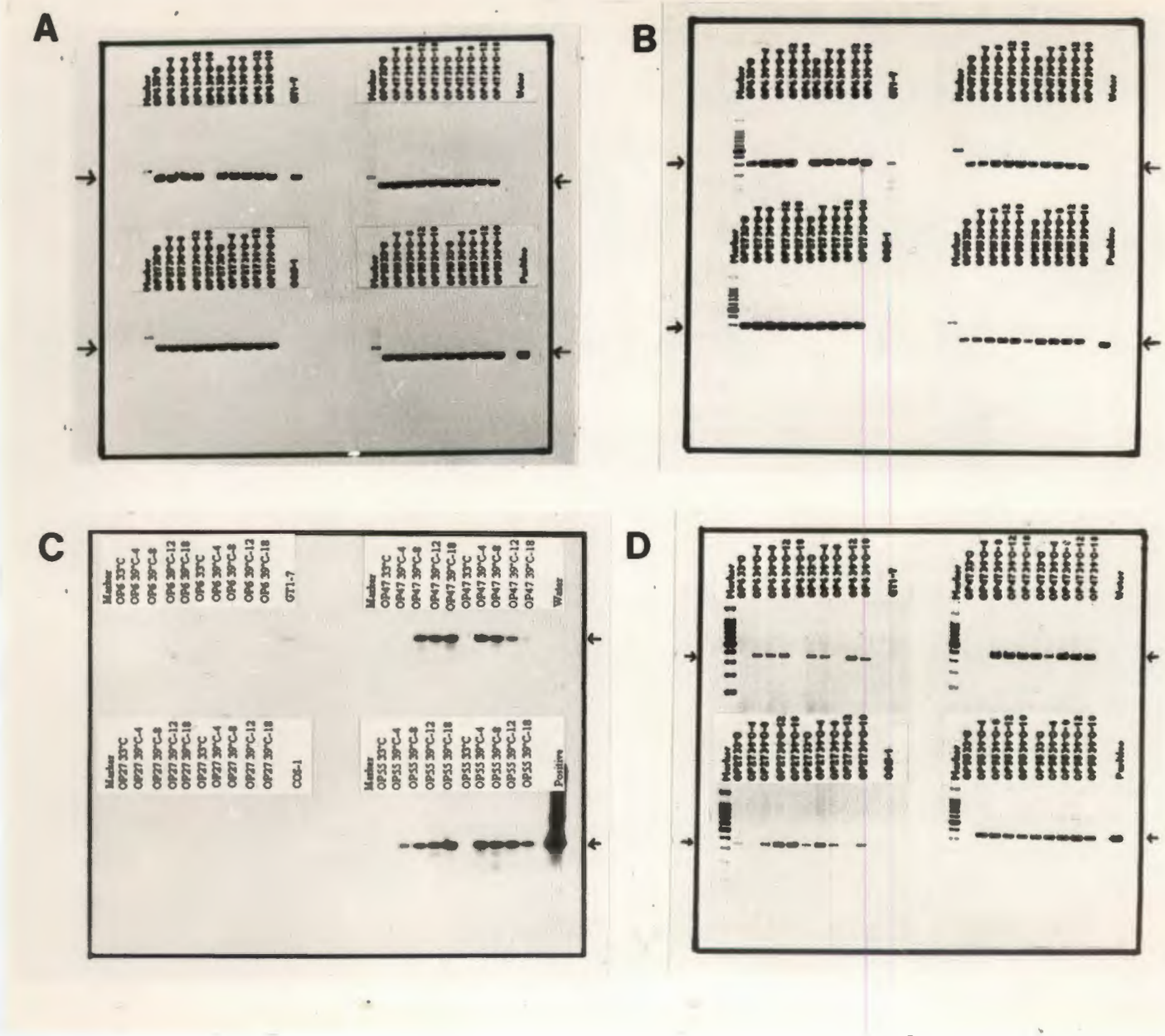


Fig 4.4 (A-D) Agarose gels (A, B, D) or autoradiographs (C) of RT-PCR products from OP cells amplified with the β-actin primers to amplify the expected size product of 364 bp (A), Olf-1 primers - 306 bp (B), Mash-1 primer- 451 bp (C) and BF-1 primers - 324 bp (D). The expected sizes are indicated by an arrow. Each cell line represents 10 samples and a marker. The lane on the left hand side represents the 100 bp DNA ladder marker. The lanes (1-5) represent the OP cells grown in DM-10 and the lanes (6-10) represented the cells grown in the RA medium for the temperature and times indicated. The numbers 4, 8, 12, 18 after the temperature, 39°C, represents the days for which the OP cell line was maintained at 39°C. The controls are indicated; GT1-7, COS-1, water and positive controls.

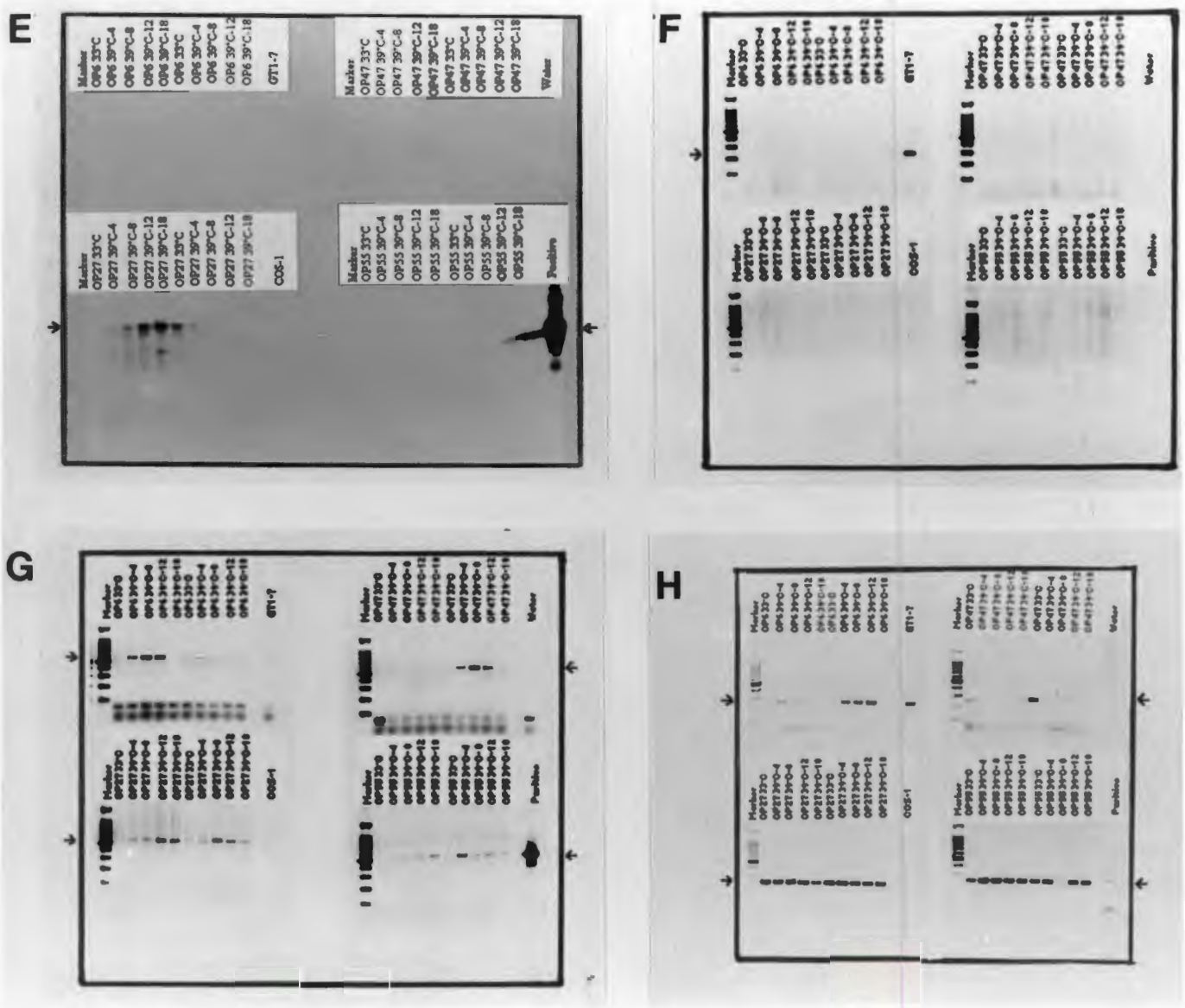


Fig 4.4 (E-H) Autoradiographs (E) or agarose gels (F, G, H) of RT-PCR products of RNA from OP cells amplified with the Pax6 primers to amplify the expected size products of 736 bp (E), GnRH primers - 241 bp (F); olfactory receptor primer - 510 bp (G) and Otx2 primers - 250 bp (H). The expected sizes are indicated by an arrow.

33°C 39°C

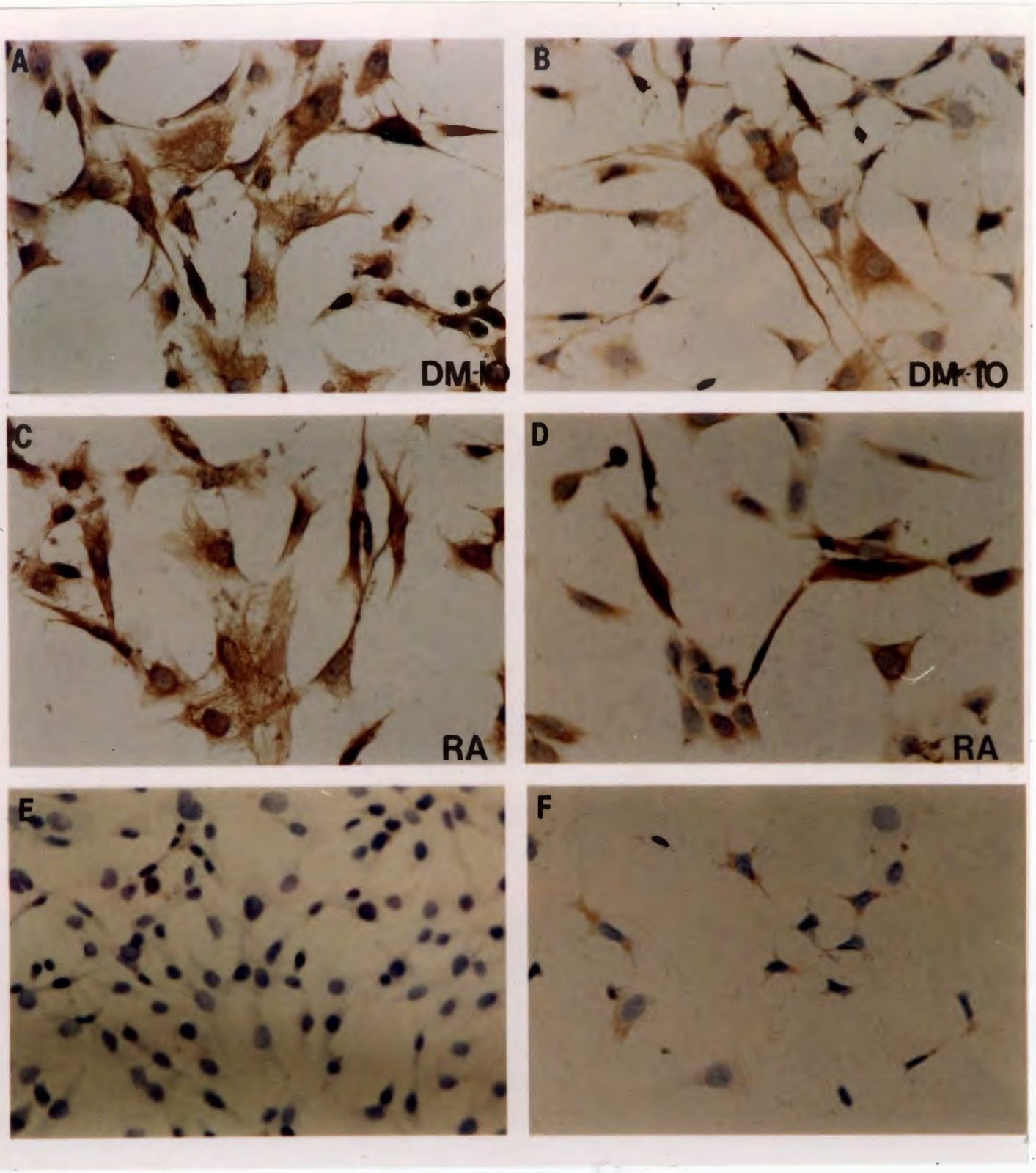


Fig 4.5 Immunocytochemistry of the OP6 cells stained with an antibody to nestin. The cells were maintained at 33°C (A, C) or at 39°C (B, D) in DM-10 medium (A, B) and in RA medium (C, D). The negative controls at 33°C (E) and at 39°C for 10 days (F) were performed exactly as for the experiment except in the absence of a primary antibody. The cells were visualised with DAB (brown precipitate) and counterstained with hematoxylin (blue nuclei) staining. Magnification 100x.

Table 4.1 Summarised results of the RT-PCR performed on RNA from OP6 cells maintained in the DM-10 and the RA medium (RA) experiments. The cells were maintained in culture at 33°C or at 39°C for 4, 8, 12, or 18 days. The expression of a particular marker is indicated by (+) or the absence of expression by (-). OR is RT-PCR for the olfactory receptor neuron transcripts.

Cell line	Medium	β-actin	Olf-1	Mash-1	BF-1	Pax6	GnRH	OR	Otx2
OP6 33°C	**DM-10**	+	+	-	-	-	-	-	-
OP6 39°C-4		+	+	-	+	-	-	+	+
OP6 39°C-8		+	+	-	+	-	-	+	-
OP6 39°C-12		+	+	-	+	-	-	+	-
OP6 39°C-18		-	-	-	-	-	-	-	-
OP6 33°C	**RA**	+	+	-	+	-	-	+	-
OP6 39°C-4		+	+	-	+	-	-	+	+
OP6 39°C-8		+	+	-	-	-	-	+	+
OP6 39°C-12		+	+	-	+	-	-	+	+
OP6 39°C-18		+	+	-	+	-	-	+	-

Table 4.2 Summarised results of the ICC on the OP6 cell lines maintained in the DM-10 and RA medium (RA). The OP cells were maintained in culture at 33°C or at 39°C for 4, 8, 12, or 18 days. The expression of a particular marker is indicated by (+++) for very strong positive staining; (bg) for background staining and (-) for the absence of stained cells. Slides in which both positive and negative cells were present, six randomly chosen fields were counted for each time point and the percentage (%) positive cells ± SD was presented instead of a symbol.

Cell line	Medium	Nestin	Vimentin	NF160	GFAP	O1	Keratin
OP6 33°C	**DM-10**	+++	-	-	-	-	3.95±1.87
OP6 39°C-4		35.33±6.4	-	-	-	-	-
OP6 39°C-8		15.83±8.5	-	-	-	-	-
OP6 39°C-12		13.72±5.60	-/bg	bg	-/bg	bg	bg
OP6 33°C	**RA**	+++	-	-	-	-	-
OP6 39°C-4		86.83±8.69	-	-/ bg	-	-	bg
OP6 39°C-8		87.00±6.23	-/bg	bg	-/ bg	bg	bg
OP6 39°C-12		82.67±6.06	-/bg	bg	-/ bg	bg	bg
OP6 39°C-18		82.33±9.71	-/bg	bg	-/ bg	bg	bg

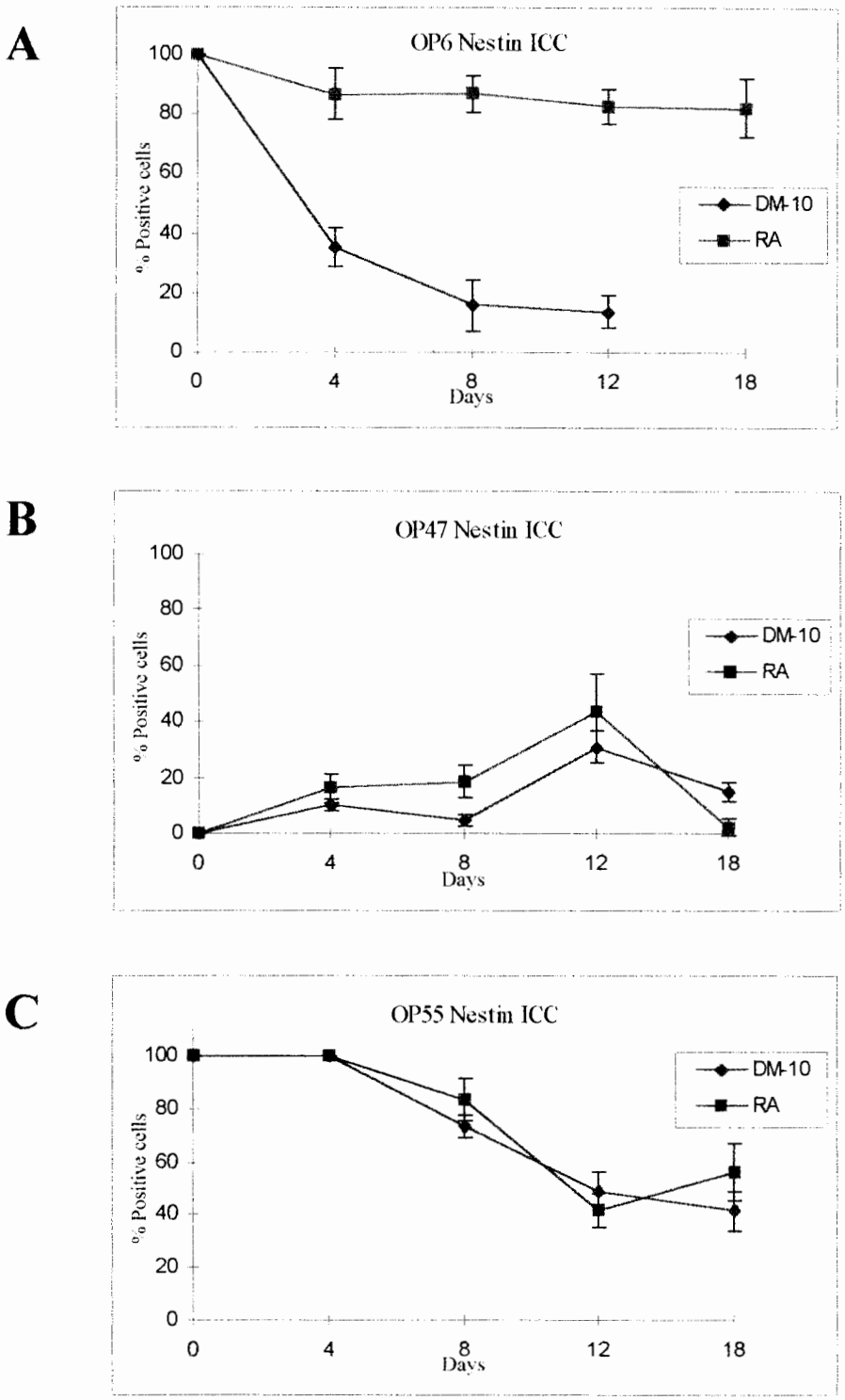


Fig 4.6 Change in nestin protein expression for OP6 (A); OP47 (B) and OP55 (C) in DM-10 and in RA medium at 33°C (day 0) and at 39°C (4, 8, 12 and 18 days). For each time point, six fields were counted and the percentage (%) ± standard deviation (SD) of positive cells plotted as a function of time.

4.3.3 *OP27 cell line*

4.3.3 (a) OP27 in DM-10 at 33°C vs. RA at 33°C

The OP27 cells maintained at 33°C in DM-10 [Fig 4.7 (A)] or the RA medium [Fig 4.8 (A)] grew as a relatively homogenous cell population of large, irregular cells (^) with numerous refractile mitoses (↟). Many of the cells were multinucleated - a feature that became more prominent the longer the cells were maintained in culture [Fig 4.7 (A)].

A change from DM-10 to RA medium at 33°C had no effect on the expression for Olf-1 [Fig 4.4 (B)], BF-1 [Fig 4.4 (D)], Otx2 [Fig 4.4 (H)] (lanes 1 & 6) transcripts which remained positive and Mash-1 and GnRH transcripts which were negative (Table 4.3). The protein expression of vimentin, NF160, GFAP, O1 and keratin also remained unchanged in both DM-10 and RA medium at 33°C (Table 4.4). ICC showed that all the cells expressed nestin at 33°C in DM-10 [Fig 4.9 (A)] and in RA [Fig 4.9 (C)].

A shift to RA medium resulted in an increase in expression of transcripts of Pax6 [Fig 4.4 (E)]. A PCR product for OR could also be detected [Fig 4.4 (G), lane 6].

4.3.3 (b) OP27 in DM-10 at 39°C

Within 4 days of shifting OP27 cells from 33°C to 39°C in DM-10, there was a noticeable change in morphology [Fig 4.7 (B)]. Although the majority of cells were still large and irregularly shaped, in many the cytoplasm diminished in size and long extensions developed that projected towards neighbouring cells (▲) in a manner similar to that observed with the OP6 cells.

After 8 days at 39°C [Fig 4.7 (C)], the cultures clearly contained two cell types: flat cells with diffusely spread, irregular, "streaky" cytoplasm (^) and increased numbers (relative to day 4) of cells with long extensions that connected with each other and formed a network across the other cells (▲).

The changes in morphology noticed in day 8 were more pronounced at day 12 [Fig 4.7 (D)]. More of the large cells had the "streaky" appearance (^) and many now appeared unhealthy. There was also an increase in the number of cells with extensions (▲).

After 18 days at 39°C [Fig 4.7 (E)] the majority of OP27 cells still appeared relatively healthy compared to OP6 cells. And the two cell morphologies remained prominent with the darker cells with long extensions (▲) forming a network across the flat cells (^) on top of which they grew.

A shift from 33°C to 39°C had no effect on the expression of Olf-1, Otx2 (which remained positive) and Mash-1, GnRH, vimentin, NF160, GFAP or O1 (which remained negative) in OP27 cells grown in DM-10 (Tables 4.1; 4.2).

For the nestin positively stained cells, the two cell types present at 39°C was very noticeable at days 4-12 [Fig 4.9 (B)]. The large, flat cells with very large nuclei had a slightly weaker staining than the smaller, elongated cells. These cells grew ontop of the large cell type. All cells remained nestin positive at 33°C and at 39°C in DM-10.

OP27 cells at 33°C in DM-10 were strongly keratin-positive [Fig 4.9 (G)]. When shifted to 39°C [Fig 4.9 (H)], the keratin immunoreactivity decreased slightly but this was difficult to interpret because of the high background staining that developed at this temeprature. This effect on background immunoreactivity was consistently observed with OP27 cells at 39°C. Other cell lines studied using the same antibody did not show this effect.

The transcripts for Pax6 and OR, which were absent at 33°C in DM-10, were amplified when OP27 was grown at 39°C in DM-10. The Pax6 transcript was faintly expressed at 39°C after 4 days and then increased and was very strongly expressed by 18 days at 39°C in the DM-10 [Fig 4.4 (E), lanes 1-5]. The transcript for the OR was detected after 4 days at 39°C, and increased gradually thereafter [Fig 4.4 (G), lanes 2-5].

BF-1 transcripts were very weakly detectable at 33°C in DM-10 and absent shortly after the shift to 39°C. Maintaining the cells longer at 39°C saw an increase once again in the BF-1 transcript - weak after 8 days and becoming stronger after 12 days at 39°C [Fig 4.4 (D)].

4.3.3 (c) OP27 in RA at 39°C

Shifting to 39°C in RA medium was followed by significant cell death with residual confluency of only 50%. This high cellular death rate was not seen with cells shifted to 39°C in DM-10 medium. The surviving cells maintained a relatively healthy appearance. The majority were large, flat and

irregular cells with jagged edges [Fig 4.8 (C)]. The longer the cells were maintained in culture, the larger their cell bodies became (∧). A small percentage (~10%) started to extend neurite-like processes [Fig 4.8 (C, D, E), ▲].

The addition of RA to the temperature shift had no effect on the expression of Olf-1, Mash-1, BF-1, GnRH and Otx2 transcripts (Table 4.3). The protein expression of vimentin, NF160, GFAP or O1 were also unchanged (Table 4.4). Olf-1, BF-1 and Otx2 transcripts were continually expressed while the other markers remained absent.

Neither shifting to 39°C alone [Fig 4.9 (B)] or in the presence of RA [Fig 4.9 (D)] had any effect on nestin immunoreactivity when compared to the cells at 33°C [Fig 4.9 (A; C)] and at 39°C in DM-10 [Fig 4.9 (B)]. Nestin protein continued to be expressed and was not down-regulated as with the other cell lines.

The keratin immunoreactivity was the same as that seen for the OP27 cells maintained at 39°C in DM-10, where there was a slight decrease in staining but the staining was masked by a high background staining on the slides [Fig 4.9 (J)].

OP27 cells maintained in the RA medium expressed Pax6 faintly at 33°C and less so when the cells were shifted to 39°C [Fig 4.4 (E)]. This differed from cells grown in DM-10, where no Pax6 transcript was detected at 33°C and a shift to 39°C resulted in a strong increase in Pax6 expression [Fig 4.4 (E)].

OR transcripts were weakly detectable at 33°C in RA medium; they increased with a shift to 39°C in RA medium, peaking on day 8. OR expression was similar to that seen in DM-10 medium at 39°C.

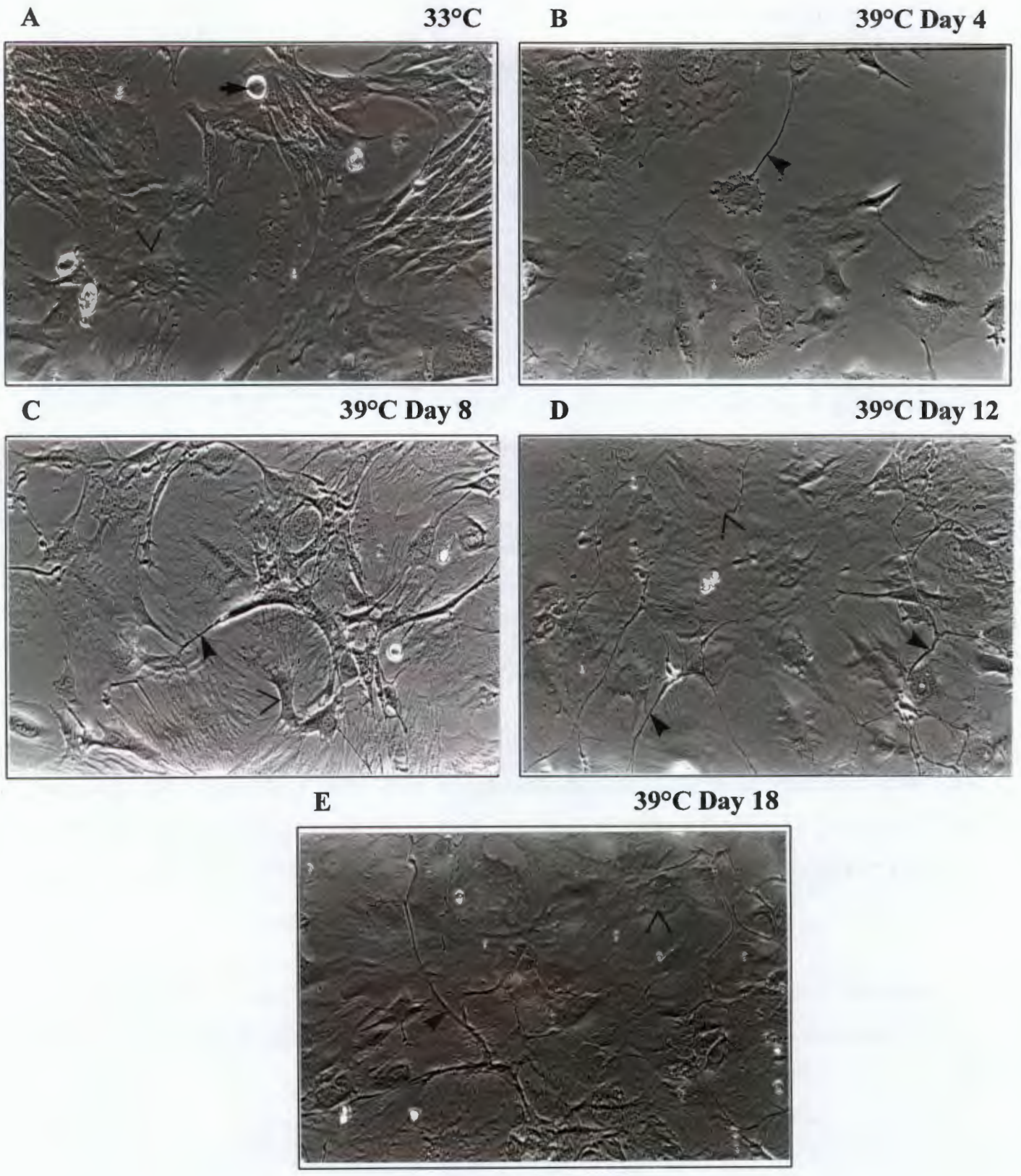


Fig 4.7 Morphology of OP27 cells maintained at 33°C or at 39°C in DM-10 medium. Phase contrast micrographs of OP27 cells at 33°C (A) or 39°C day 4 (B), day 8 (C), day 12 (D), day 18 (E). Cultures were photographed with an inverted microscope at 50x magnification. ∧ represents large, flat epithelial-like cells, ▲ represents cells with extensions and ↟ represents refractile mitosis. The morphology is discussed in the results section.

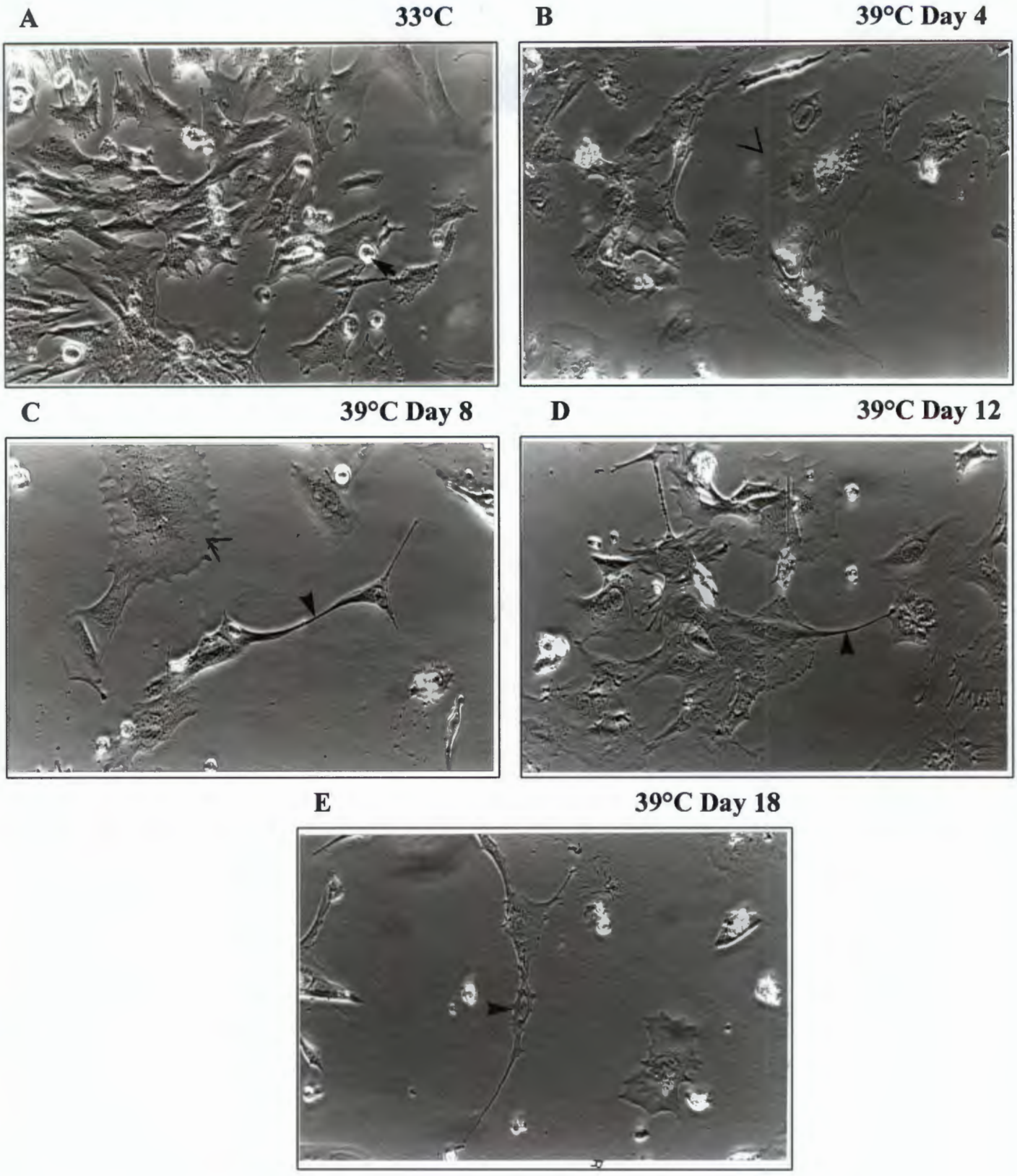


Fig 4.8 Morphology of OP27 cells maintained at 33°C or at 39°C in RA medium. Phase contrast micrographs of OP27 cells at 33°C (A) or 39°C day 4 (B), day 8 (C), day 12 (D), day 18 (E). Cultures were photographed with an inverted microscope at 50x magnification. ∧ represents large, flat epithelial-like cells, ▲ represents cells with extensions and ↟ represents refractile mitosis. The morphology is discussed in the results section.

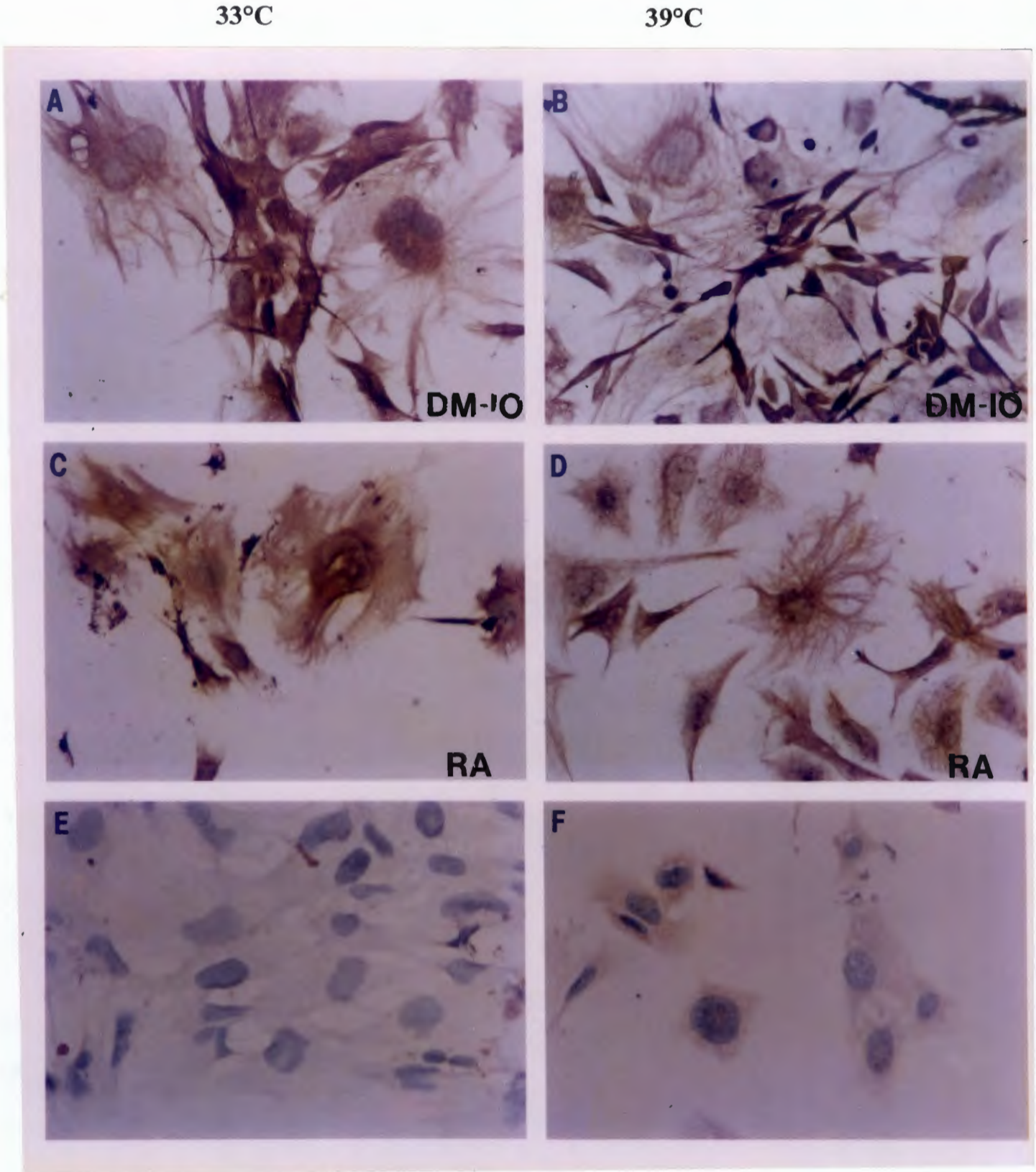


Fig 4.9 (A-F) Immunocytochemistry of OP27 cells stained with an antibody to nestin (A-F). Cells were maintained at 33°C (A, C, E) or 39°C (B, D, F) in DM-10 (A, B) or RA medium (C, D). The negative controls at 33°C (E) and 39°C for 10 days (F) were performed as for the experiments except in the absence of a primary antibody. Cells were visualised with DAB (brown) and counterstained with hematoxylin (blue nuclei). 100x magnification.

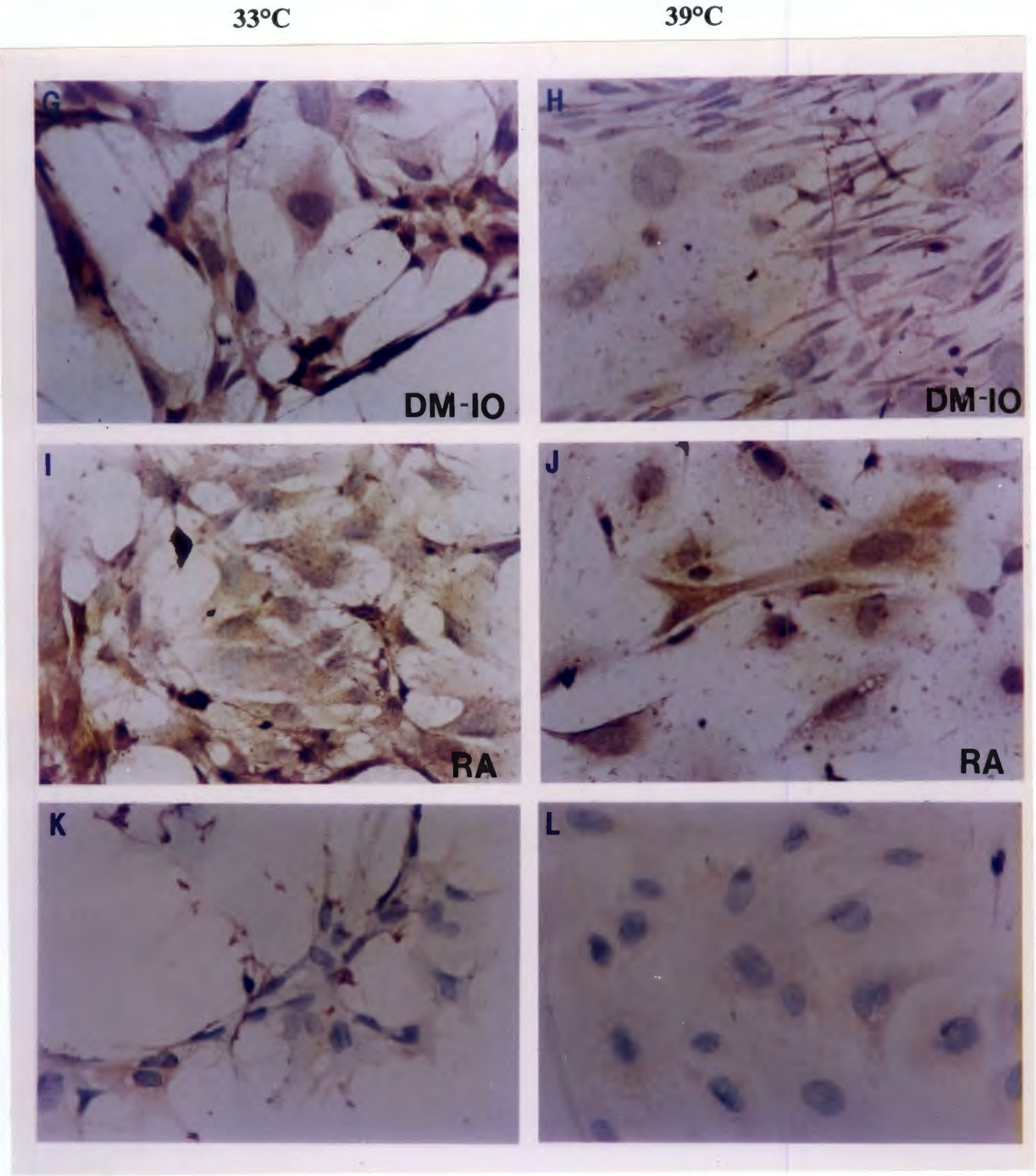


Fig 4.9 (G-L) Immunocytochemistry of OP27 cells stained with an antibody to keratin (G-L). Cells were maintained at 33°C (G, I, K) or 39°C (H, J, L) in DM-10 (G, H) or RA medium (I, J). The negative controls at 33°C (K) and 39°C for 10 days (L) were performed as for the experiments except in the absence of a primary antibody. Cells were visualised with DAB (brown) and counterstained with hematoxylin (blue nuclei). 100x magnification.

Table 4.3 Summarised results of the RT-PCR performed on RNA from OP27 cells maintained in the DM-10 medium and the RA medium (RA) experiments. The OP27 cells were maintained in culture at 33°C or at 39°C for 4, 8, 12, or 18 days. The expression of a particular marker is indicated by (+) or the absence of expression by (-). OR is RT-PCR for the olfactory receptor neuron transcripts.

Cell line	Medium	β-actin	Olf-1	Mash-1	BF-1	Pax6	GnRH	OR	Otx2
OP27 33°C	**DM-10**	+	+	-	+	-	-	-	+
OP27 39°C-4		+	+	-	-	+	-	+	+
OP27 39°C-8		+	+	-	+	+	-	+	+
OP27 39°C-12		+	+	-	+	+	-	+	+
OP27 39°C-18		+	+	-	+	+	-	+	+
OP27 33°C	**RA**	+	+	-	+	+	-	+	+
OP27 39°C-4		+	+	-	+	+	-	+	+
OP27 39°C-8		+	+	-	+	-	-	+	+
OP27 39°C-12		+	+	-	+	-	-	+	+
OP27 39°C-18		+	+	-	+	-	-	+	+

Table 4.4 Summarised results of the ICC on the OP27 cell lines maintained in the DM-10 and RA medium (RA). The OP27 cells were maintained in culture at 33°C or at 39°C for 4, 8, 12, or 18 days. The expression of a particular marker is indicated by (+++) for very strong positive staining; (++) for weaker positive staining; (bg) for background staining and (-) for the absence of stained cells. Slides in which both positive and negative cells were present, six randomly chosen fields were counted for each time point and the percentage (%) positive cells ± SD was presented instead of a symbol.

Cell line	Medium	Nestin	Vimentin	NF160	GFAP	O1	Keratin
OP27 33°C	**DM-10**	+++	-	-/ bg	-	-	+++
OP27 39°C-4		+++	-	-/ bg	-	-	++
OP27 39°C-8		+++	-	-/ bg	-	-	++
OP27 39°C-12		+++	-	-/ bg	-	-	++
OP27 39°C-18		+++	-	-/ bg	-	-	++
OP27 33°C	**RA**	+++	-	-/ bg	-	-	+++
OP27 39°C-4		+++	-	-/ bg	-	-	++
OP27 39°C-8		+++	-/ bg	-/ bg	-/ bg	-/ bg	++
OP27 39°C-12		+++	-	-/ bg	-/ bg	-/ bg	++
OP27 39°C-18		+++	-	-/ bg	-/ bg	-/ bg	++

4.3.4 OP47 cell line

4.3.4 (a) OP47 in DM-10 at 33°C vs. RA at 33°C

At 33°C RA medium [Fig 4.11 (A)] had no effect on the morphology of OP47 cells with a shift from DM-10 [Fig 4.10 (A)]. In the RA medium the photomicrograph was taken of a more confluent culture and the cells are packed closer and thus appears smaller than those in the DM-10 medium. At 33°C under both conditions, the OP47 cells grew as a population of large, irregular shaped cells with numerous refractile mitosis.

The expression of Olf-1 [Fig 4.4 (B)] and Otx2 [Fig 4.4 (H)] remained positive at 33°C in both the DM-10 and the RA medium. No protein expression nor mRNA transcripts for vimentin, NF160, GFAP, O1, keratin, Mash-1, Pax6 , GnRH or OR were detected (Tables 4.5; 4.6).

BF-1 transcripts were not detected in DM-10 at 33°C. In the RA medium this gene was expressed strongly [Fig 4.4 (D); Table 4.5]. Nestin immunoreactivity was undetected both in DM-10 and in RA at 33°C [Fig 4.12 (A; C)].

4.3.4 (b) OP47 in DM-10 at 39°C

After 4 days at 39°C in DM-10 [Fig 4.10 (B)], most of the cells resembled those seen at 33°C with, in addition, a few cells starting to produce long, thin processes that extended, in bipolar fashion, from small cell bodies and ended by contacting other cells (▲).

After 8 days at 39°C [Fig 4.10 (C)], there were many cells with extensions that varied individually in length and projected, as unipolar or bipolar processes from thin, elongated cell bodies (▲). As with OP6 and OP27 cells, these cells with extensions grew on top of a second larger, irregular cell type (∧). From day 12 onwards these flat cells (∧) started to look sick whereas the other cells maintained a healthy appearance with extensions that grew longer (▲) (in some cases reaching 3-4x's the length of the cell body) with time [Fig 4.10 (D)].

Even after 18 days at 39°C [Fig 4.10 (E)] most of the cells looked reasonably healthy. As in the case of the other cell lines, the cells with extensions (▲) grew on top a flat, irregular underlayer

shaped cells (∧) but they did not form the characteristic network-like structures noticed with OP6 and OP27 at 39°C.

OP47 cells survived for 3 weeks at 39°C and they remained healthy provided they were supplied with fresh medium.

Shifting from 33°C to 39°C in DM-10 did not induce expression of vimentin, NF160, GFAP, keratin, Pax6 or GnRH (Tables 4.6; 4.5). Olf-1 expression, consistently positive throughout the experiment, remained so without change [Fig 4.4 (B)].

Cells were nestin-negative at 33°C. A shift to 39°C resulted in an increase in protein expression which was strongly expressed in some cells while other cells remained negative [Fig 4.12 (B)]. Nestin expressing cells constituted 10% of the population at day 4, decreasing to 5% later then increasing again [Fig 4.6 (B); Table 4.6].

Mash-1, BF-1 and OR mRNAs were not detected at 33°C. Mash-1 [Fig 4.4 (C)] and BF-1 [Fig 4.4 (D)] transcripts were strongly expressed after 8 days at 39°C and remained so until the end of the experiment. OR transcript appeared after 4 days at 39°C and remained constantly very faint throughout the time at 39°C [Fig 4.4 (G)].

Otx2 expression differed completely from that observed in the other cell lines in that it was only evident at 33°C. Expression ceased once the cells were shifted to 39°C [Fig 4.4 (H); Table 4.5].

The OP47 cells were negative for O1 at 33°C in the DM-10 [Fig 4.12 (G)] but a shift to 39°C resulted in an equivocal increase in immunoreactivity for O1 marker from day 12 [Fig 4.12 (H)] of dubious significance (Table 4.6).

4.3.4 (c) OP47 in RA at 39°C

The OP47 cells unlike the OP6 and the OP27 cell lines, did not die when shifted to 39°C in the presence of RA and their cell number remained constant at a confluency of 80-90% throughout the experiment. At 39°C their cell bodies became elongated [Fig 4.11 (B), ▲]. After 8 days of culture [Fig 4.11 (C)] two morphological types were clearly distinguishable. The predominating cells were flat and irregular in shape with a large cytoplasm:nucleus ratio (∧). Positioned on top of these flat

cells was a smaller population of cells with small, round cell bodies and long extensions that made contact with other cell extensions [▲, Fig 4.11 (C, D, E)]. These bipolar processes became longer as the cells were maintained in culture.

There was no significant difference between the OP47 cells maintained in the RA medium and those maintained in the DM-10 medium.

When the OP47 cells were grown in RA medium at 39°C, there was no change in the expression of vimentin, NF160, GFAP, keratin, Olf-1, Pax6 and GnRH as compared to cells grown in DM-10 at 39°C (Tables 4.6; 4.5).

At 33°C in both DM-10 and in RA medium, Mash-1 transcripts were undetected. A shift to 39°C in both growth conditions resulted in the up-regulation of Mash-1 transcripts [Fig 4.4 (C)].

The BF-1 transcript was expressed strongly in all samples in the RA medium at both 33°C and at 39°C whereas in DM-10 transcription was only seen after 8 days at 39°C [Fig 4.4 (D)].

The OP47 cells did not contain OR transcripts at 33°C in either DM-10 or in RA medium. When shifted to 39°C in DM-10, a faint transcript was detected by RT-PCR. However, when shifted to 39°C in the RA medium, OP47 cells expressed the transcript weakly on day 4 and then its expression peaked on day 8. Thereafter expression decreased [Fig 4.4 (G)].

The Otx2 transcript was present at 33°C both in DM-10 and in RA medium but stronger in the RA medium. A shift to 39°C under both conditions resulted in the immediate decrease in Otx2 expression [Fig 4.4 (H)].

The O1 marker was not expressed by OP47 cells in the RA medium, although there was a high background staining the longer the cells were maintained at 39°C and this background staining was also detected at 39°C in the negative control (Table 4.6).

ICC showed that nestin protein was absent at 33°C in DM-10 and in RA medium [Fig 4.12 (G; I)] but a shift to 39°C, resulted in an increase in expression of nestin positive cells [Fig 4.12 (B; D)]. The percentage of positive cells was in the order of 16-40% with a peak expression at day 12 [Fig 4.6 (B); Table 4.6].

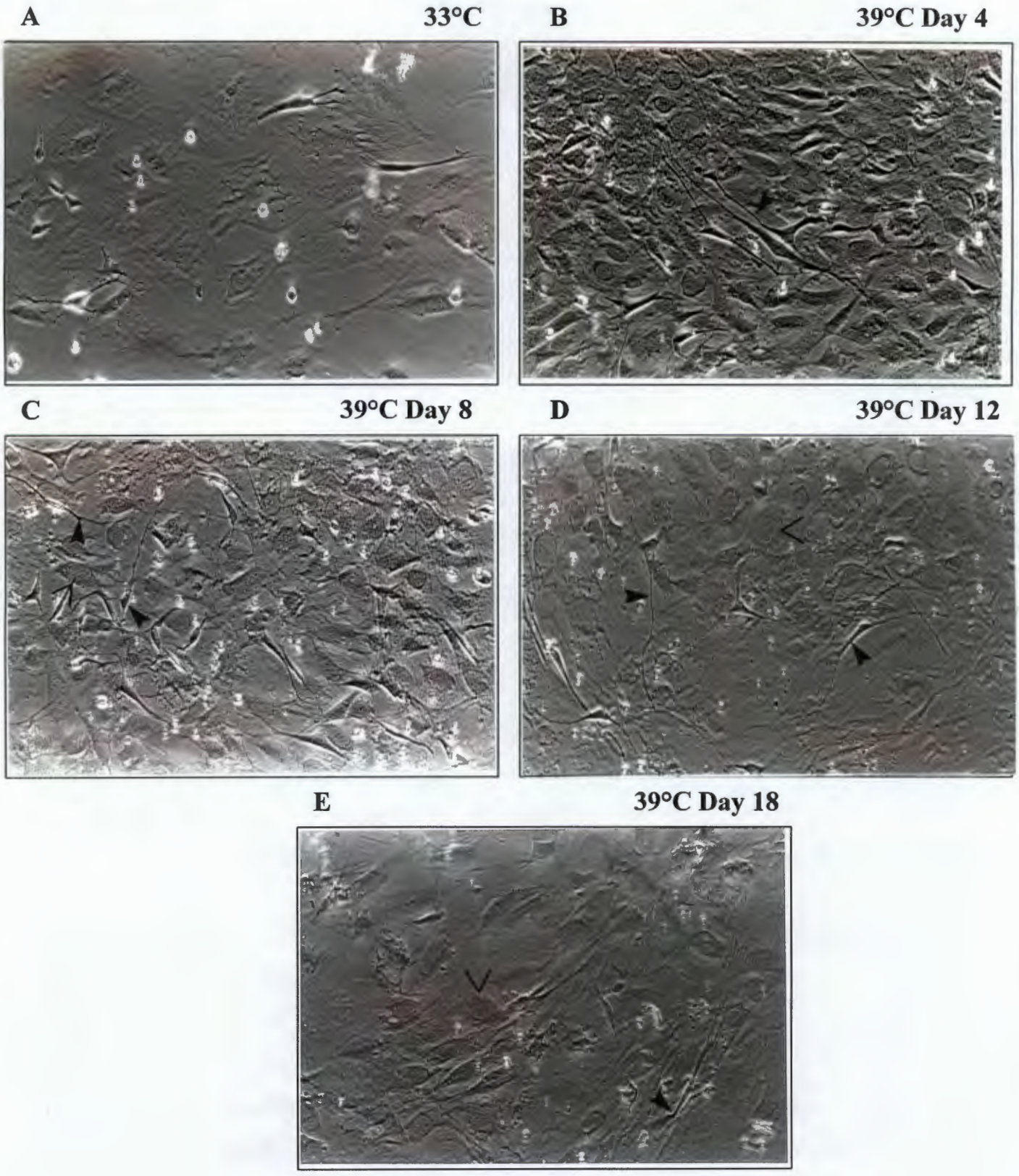


Fig 4.10 Morphology of OP47 cells maintained at 33°C or at 39°C in DM-10 medium. Phase contrast micrographs of OP47 cells at 33°C (A) or 39°C day 4 (B), day 8 (C), day 12 (D), day 18 (E). Cultures were photographed with an inverted microscope at 50x magnification. ∧ represents large, flat, epithelial-like cells and ▲ represents cells with extensions. The morphology is discussed in the results section.

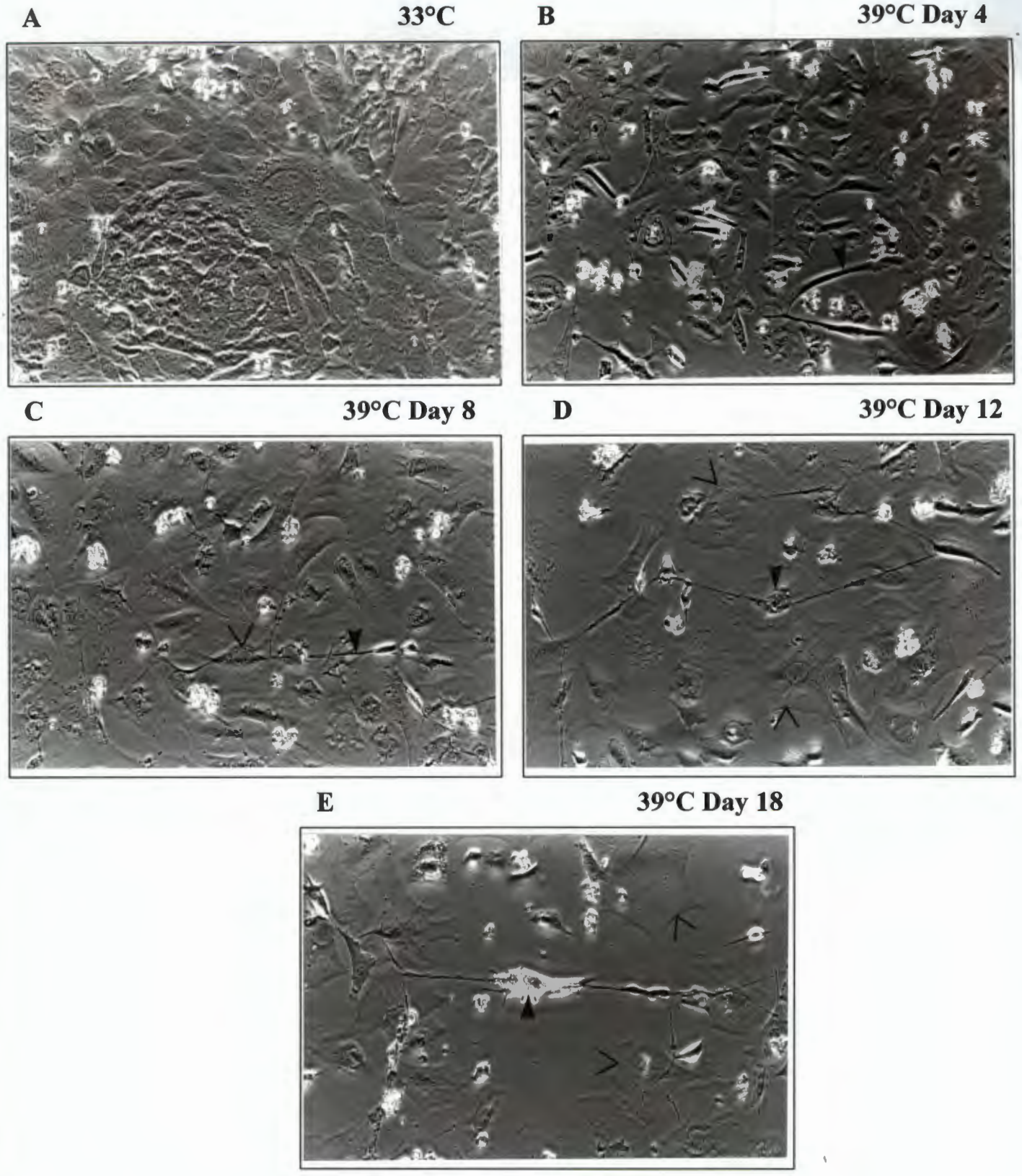


Fig 4.11 Morphology of OP47 cells maintained at 33°C or at 39°C in RA medium. Phase contrast micrographs of OP47 cells at 33°C (A) or 39°C day 4 (B), day 8 (C), day 12 (D), day 18 (E). Cultures were photographed with an inverted microscope at 50x magnification. ∧ represents large, flat, epithelial-like cells and ▲ represents cells with extensions. The morphology is discussed in the results section.

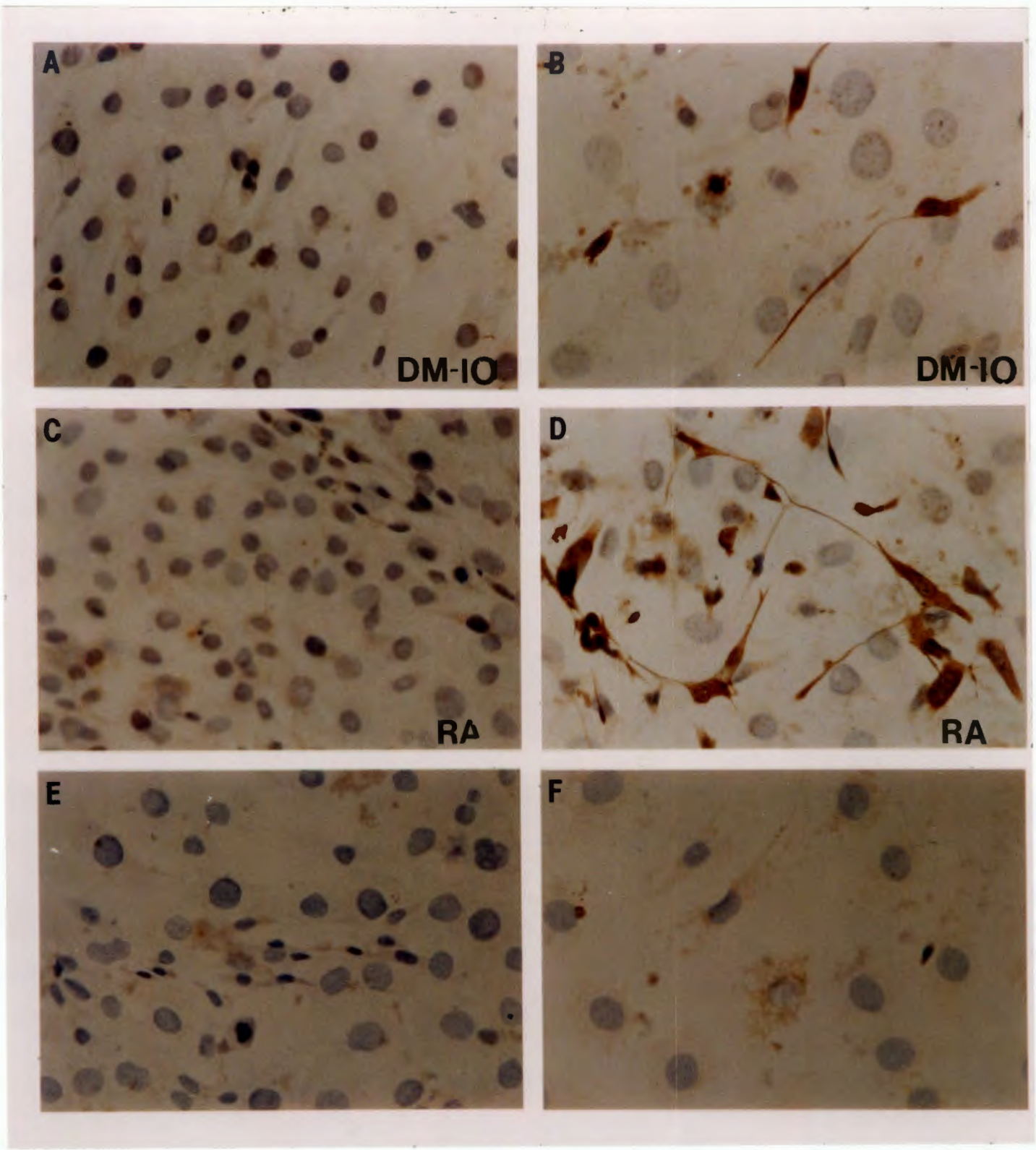


Fig 4.12 Immunocytochemistry of OP47 cells stained with an antibody to nestin. Cells were maintained at 33°C (A, C) or 39°C (B, D) in DM-10 (A, B) or RA medium (C, D). The negative controls at 33°C (E) and at 39°C for 10 days (F) omitted the primary antibody. Cells were visualised with DAB (brown) and counterstained with hematoxylin (blue nuclei). 100x magnification.

Table 4.5 Summarised results of the RT-PCR performed on RNA from OP47 cells maintained in the DM-10 and RA medium (RA). The OP47 cells were maintained in culture at 33°C or at 39°C for 4, 8, 12, or 18 days. The expression of a particular marker is indicated by (+) or the absence of expression by (-). OR is RT-PCR for the olfactory receptor neuron transcripts.

Cell line	Medium	β-actin	Olf-1	Mash-1	BF-1	Pax6	GnRH	OR	Otx2
OP47 33°C	**DM-10**	+	+	-	-	-	-	-	+
OP47 39°C-4		+	+	-	-	-	-	+	-
OP47 39°C-8		+	+	+	+	-	-	+	-
OP4739°C-12		+	+	+	+	-	-	+	-
OP47 39°C-18		+	+	+	+	-	-	+	-
OP47 33°C	**RA**	+	+	-	+	-	-	-	+
OP47 39°C-4		+	+	+	+	-	-	+	-
OP47 39°C-8		+	+	+	+	-	-	+	-
OP47 39°C-12		+	+	+	+	-	-	+	-
OP47 39°C-18		+	+	+	+	-	-	+	-

Table 4.6 Summarised results of the ICC on the OP47 cells maintained in the DM-10 and RA medium (RA). The OP47 cells were maintained in culture at 33°C or at 39°C for 4, 8, 12, or 18 days. The expression of a particular marker is indicated by (+++) for very strong positive staining; (++) for weaker positive staining; (bg) for background staining and (-) for the absence of stained cells. Slides in which both positive and negative cells were present, six randomly chosen fields were counted for each time point and the percentage (%) positive cells ± SD was presented instead of a symbol.

Cell line	Medium	Nestin	Vimentin	NF160	GFAP	O1	Keratin
OP47 33°C	**DM-10**	-	-	-	-	-	-
OP47 39°C-4		10.32±2.2	-	-	-	-	-
OP47 39°C-8		4.52±2.13	-	-	-	-	-
OP4739°C-12		30.92±5.83	-	-/ bg	-/ bg	++	-
OP47 39°C-18		15.02±3.38	-	-/ bg	-/ bg	+++	-
OP47 33°C	**RA**	-	-	-	-	-	-
OP47 39°C-4		16.17±5.08	-	-	-	-	-
OP47 39°C-8		18.58±5.68	-	-	-/ bg	-/ bg	-
OP47 39°C-12		43.50±13.58	-	-/ bg	-/ bg	-/ bg	-
OP47 39°C-18		2.33±3.01	-/ bg	-/ bg	-/ bg	-/ bg	bg

4.3.5 *OP55 cell line*

4.3.5 (a) OP55 in DM-10 at 33°C vs. RA at 33°C

The OP55 cells grown at 33°C in DM-10 medium [Fig 4.13 (A)] were similar to those grown in RA medium [Fig 4.14 (A)]. They were irregular in shape with no extensions and unlike the other cell lines that grew as single cells evenly distributed over the tissue culture surface, they tended to grow in groups or clumps.

RA at 33°C had no effect on the expression of vimentin, NF160, GFAP, Mash-1, Pax6, GnRH and OR (which remained negative) or Olf-1, Otx2, nestin and keratin (which remained positive) (Tables 4.7; 4.8).

The transcript for BF-1 was absent at 33°C in DM-10 but RA resulted in the strong expression of this transcript [Fig 4.4 (D)].

4.3.5 (b) OP55 in DM-10 at 39°C

When OP55 cells in DM-10 were shifted to 39°C, numerous mitoses (↟) were still evident after 4 days but most of the cells appeared smaller and grew as a contiguous "paved" monolayer [Fig 4.13 (B)]. A few cells had short extensions that bridged narrow gaps between adjacent cell groups (▲).

The OP55 cells maintained a constant cell density at 39°C. This was obvious from the growth curves [Fig 3.11 (D)] and on visual assessment. After 8 days at 39°C [Fig 4.13 (C)], they appeared much as they had done after 4 days.

By 12 days at 39°C [Fig 4.13 (D)], a striking morphological change became apparent with two distinct cell types now seen. The first comprised the small, compact epithelial-like cells (⇑) and the second cell type belonged to a population of elongated cells (▲) that grew in a reticular formation interspersed with mitoses (↟).

After 18 days in culture at 39°C [Fig 4.13 (E)] mitoses were sparse, the cells became flattened and cellular aggregates made their appearance as islands of closely packed, small cells (⇑) separated by large, flattened cells [∧, Fig 4.13 (F)].

OP55 differed significantly in morphology from any of the other OP cell lines grown in culture. The closely packed small cells observed in OP55 were not observed in the other cell lines and the OP55 cells did not develop cells with long "neurite-like" extensions which connected with other cells in culture.

Shifting OP55 cells grown in DM-10 from 33°C to 39°C had no effect on the expression of Olf-1, Pax6, GnRH, Otx2, vimentin, NF160, GFAP or O1 [Tables 4.7; 4.8).

Nestin immunoreactivity was strong in the OP55 cells at 33°C [Fig 4.15 (A)] and at 39°C on day 4. Thereafter expression decreased to about 74% on day 8 and to 49-41% on days 12 to 18 [Table 4.8; Fig 4.6 (C)]. Both the large flat cells and the smaller cells present in culture on day 8 were stained with anti-nestin antibody, the larger cells being more strongly immunoreactive. With the passage of time in culture, staining of the large cells diminished in intensity so that by 18 days at 39°C it was no longer detectable [Fig 4.15 (B)]. The smaller cells, however, expressed nestin throughout.

Mash-1, BF-1 or OR transcripts were not seen at 33°C in DM-10. The shift to 39°C resulted in an increase in expression that was detected as early as day 4 (Table 4.7). Mash-1 expression increased gradually peaking by day 18 at 39°C (Fig 4.6); BF-1 transcripts were equally prominent at all time points [Fig 4.4 (D)]; OR transcripts increased slightly in amount with the strongest expression seen at day 12 [Fig 4.4 (G)].

OP55 cells grown in DM-10 at 33°C expressed the antigen for keratin [Fig 4.15 (G)]. Once shifted to 39°C [Fig 4.15 (H)] the cells lost keratin immunoreactivity, becoming negative with a faint background staining [Fig 4.15 (L)].

4.3.5 (c) OP55 in RA at 39°C

When the OP55 cells were shifted to 39°C in the presence of RA many of the cells detached and the cell number decreased to about 60% confluency remaining relatively constant at this density thereafter. The cells, at 39°C, were small and irregular in shape. There were a few clumps of phase-bright rounded mitotic cells (↟) that remained attached to the tissue culture plate, similar to the cells maintained in the DM-10 medium [Fig 4.13 (D)]. With the passage of time in culture some cells enlarged and flattened (∧) while others became grew long, thin extensions towards other cells in

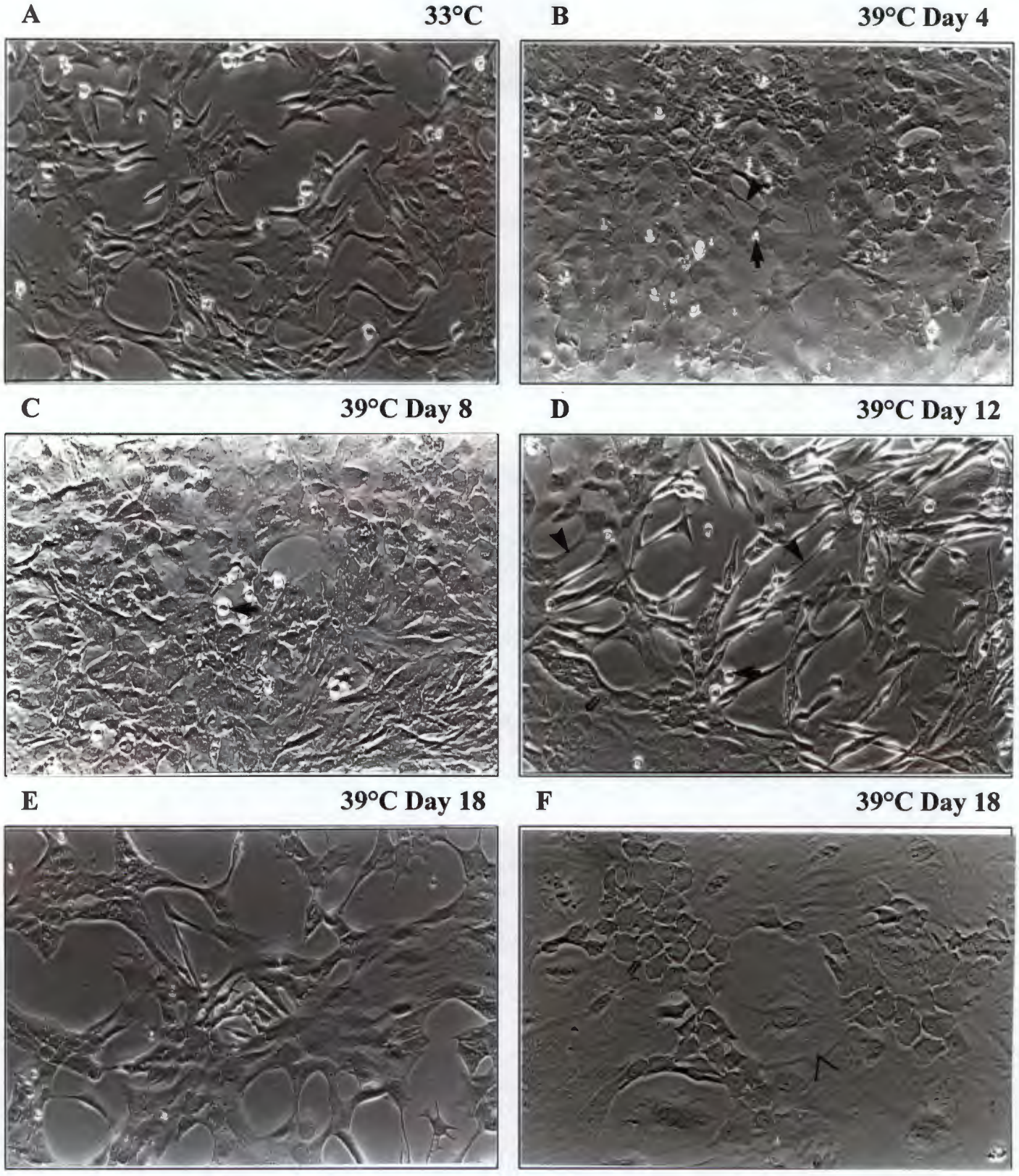


Fig 4.13 Morphology of OP55 cells maintained at 33°C or at 39°C in DM-10 medium. Phase contrast micrographs of OP55 cells at 33°C (A) or 39°C day 4 (B), day 8 (C), day 12 (D), day 18 (E). Cultures were photographed with an inverted microscope at 50x magnification. ∧ represents large, flat epithelial-like cells, ▲ represents cells with extensions, ↟ represents refractile mitosis and ⇑ represents compact epithelial cells. The morphology is discussed in the results section.

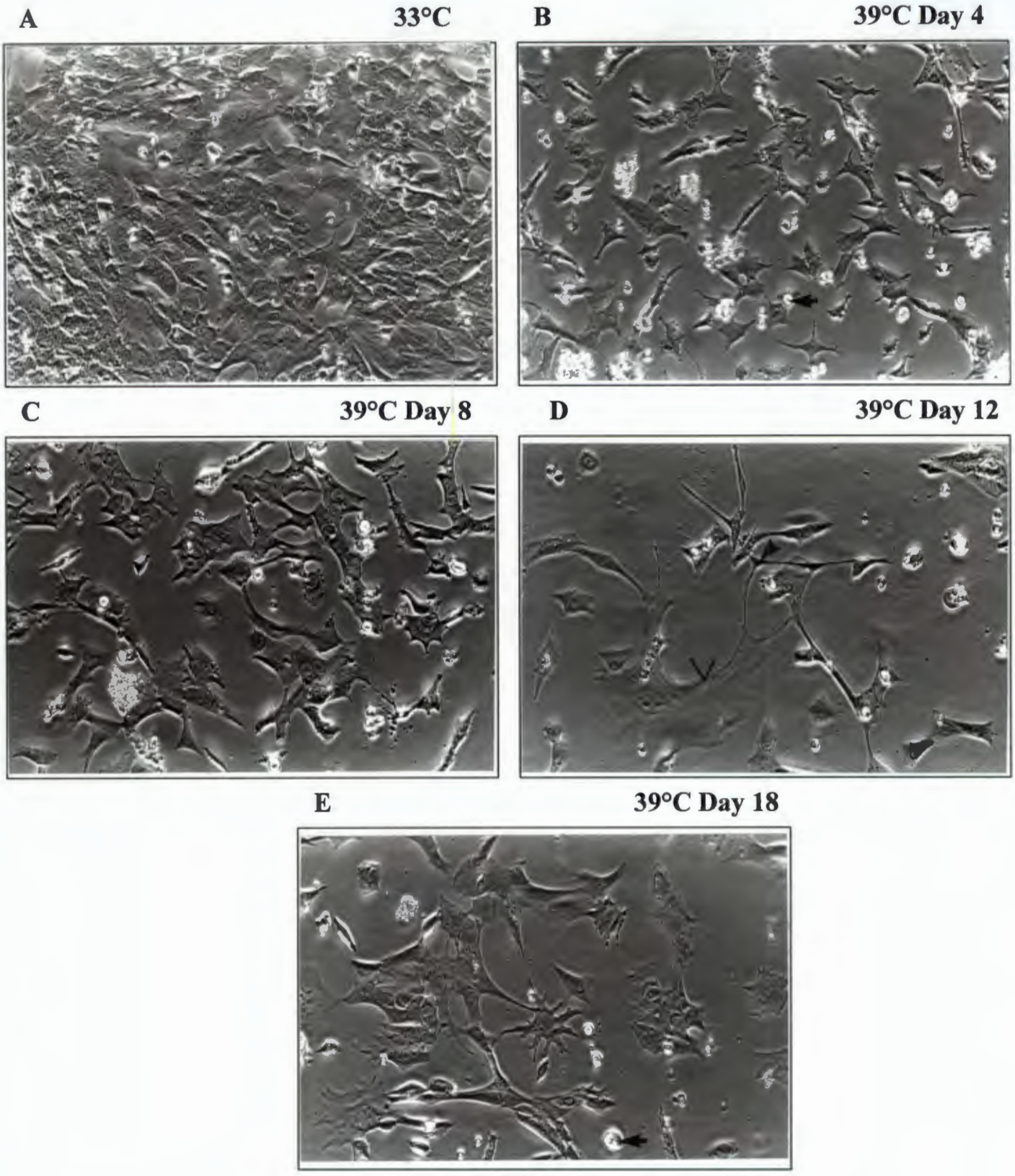


Fig 4.14 Morphology of OP55 cells maintained at 33°C or at 39°C in RA medium. Phase contrast micrographs of OP55 cells at 33°C (A) or 39°C day 4 (B), day 8 (C), day 12 (D), day 18 (E). Cultures were photographed with an inverted microscope at 50x magnification. ∧ represents large, flat epithelial-like cells, ▲ represents cells with extensions and ♦ represents refractile mitosis. The morphology is discussed in the results section.

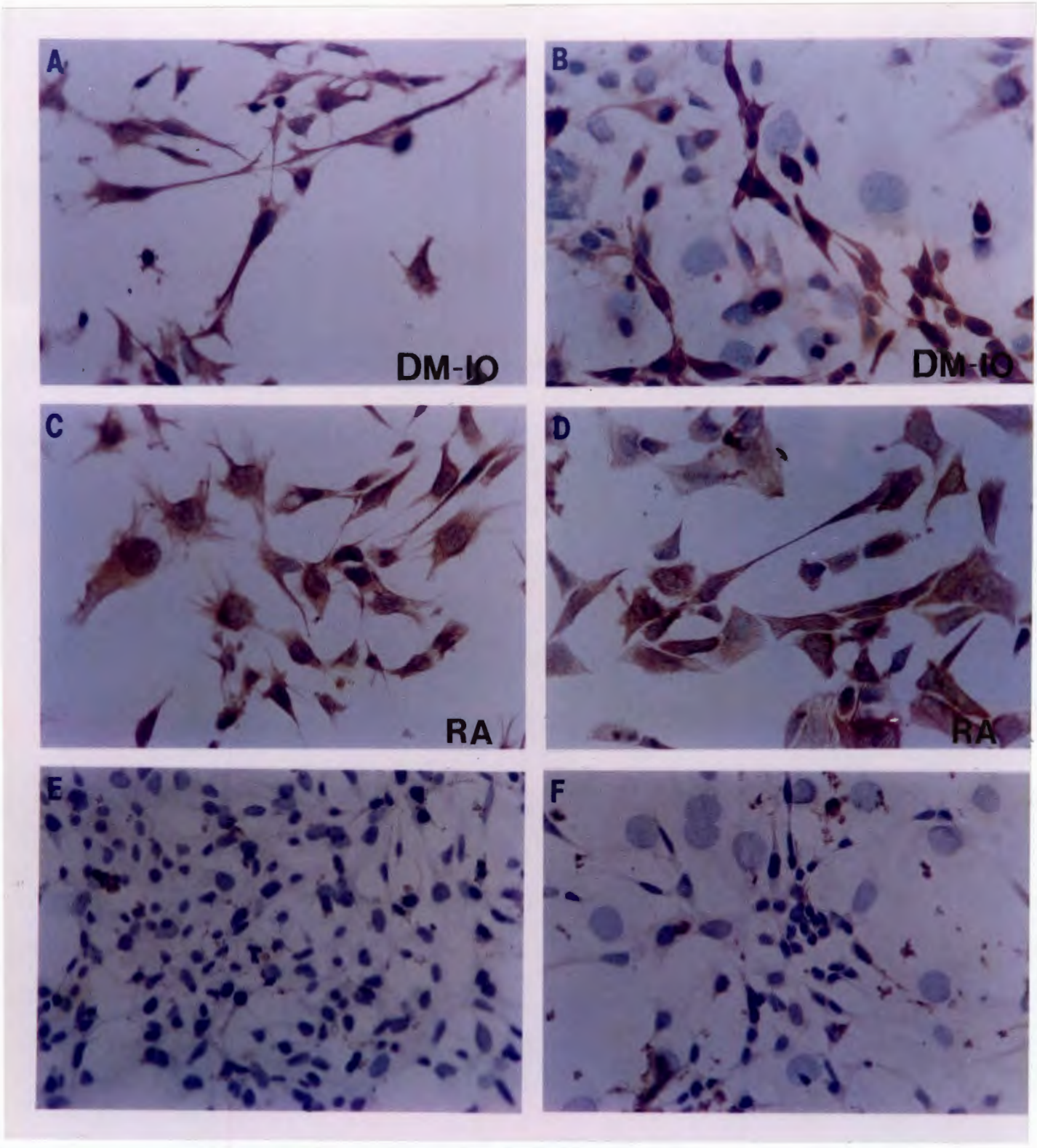


Fig 4.15 (A-F) Immunocytochemistry of OP55 cells stained with an antibody to nestin. Cells were maintained at 33°C (A, C) or 39°C (B, D) in DM-10 (A, B) or RA medium (C, D). The negative controls at 33°C (E) or 39°C for 10 days (F) were performed as for the experiments except in the absence of primary antibody. Cells were visualised with DAB (brown) and counterstained with hematoxylin (blue nuclei). 100x magnification.

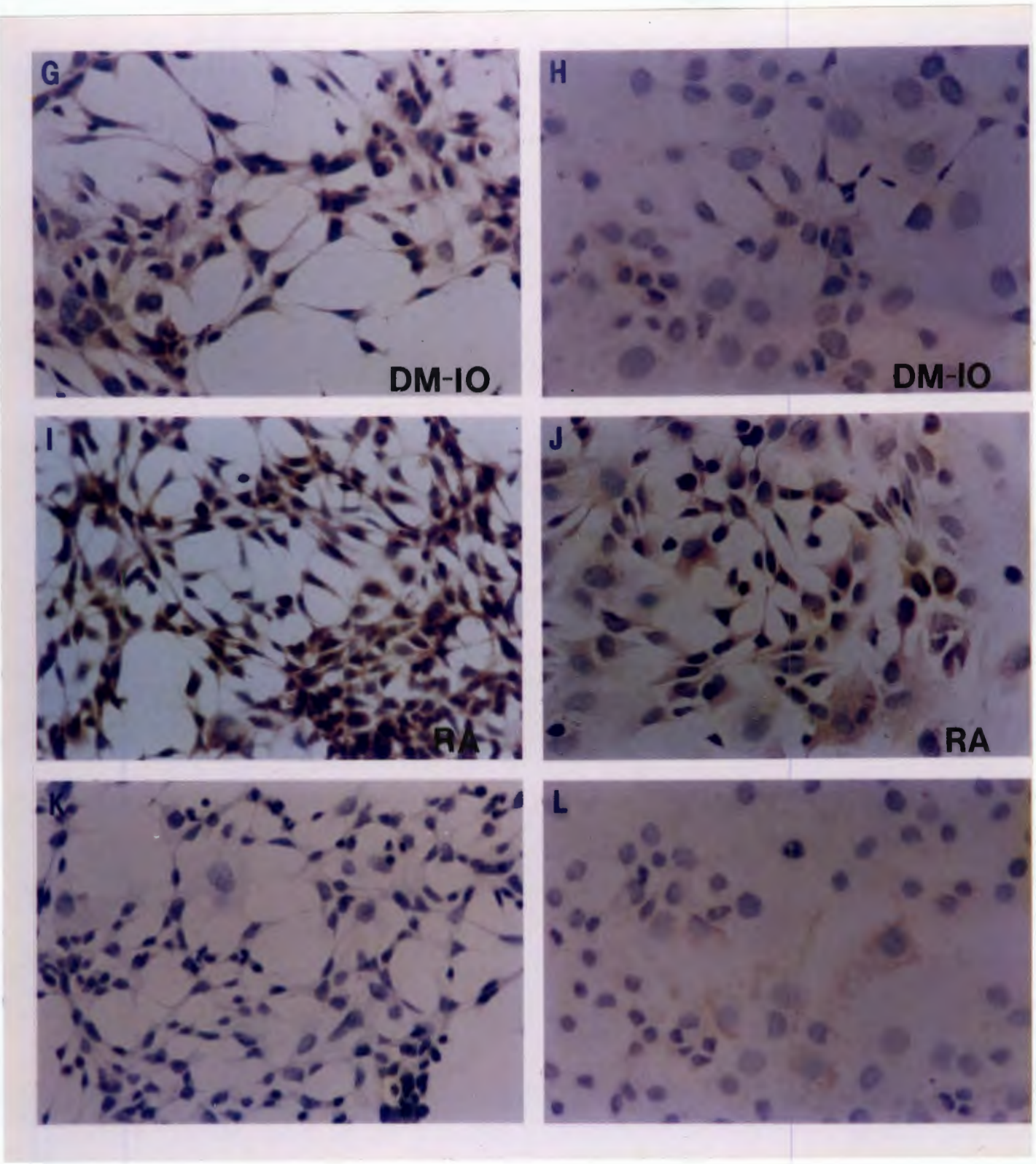


Fig 4.15 (G-L) Immunocytochemistry of OP55 cells stained with an antibody to keratin. Cells were maintained at 33°C (G, I, K) or 39°C (H, J, L) in DM-10 (G, H) or RA medium (I, J). The negative controls at 33°C (K) and 39°C for 10 days (L) omitted the primary antibody. Cells were visualised with DAB (brown) and counterstained with hematoxylin (blue nuclei). 100x magnification.

culture [▲, Fig 4.14 (D)]. All cells in culture extended their cell bodies such that they made contact with surrounding cells [Fig 4.14 (E)]. The grouping or clumping that were obvious in OP55 cell culture at 39°C in the DM-10 medium [Fig 4.13 (F)] were not seen in the RA medium. The OP55 cells maintained in the RA medium appeared less healthier than those grown in DM-10 medium.

Shifting from 33°C to 39°C, in the presence of RA, did not increase or decrease expression of Olf-1, BF-1 or Otx2. Neither Pax6, GnRH, vimentin, NF160, GFAP or O1 were expressed in either conditions. Furthermore, there was no change in the protein expression for keratin in comparison to cells in RA at 33°C (Tables 4.7; 4.8).

Keratin immunoreactivity, which was positive at 33°C in both DM-10 and in RA medium, was lost when the cells were shifted to 39°C in DM-10 but continued to be expressed at 39°C in RA medium [Fig 4.16 (J)].

Immunoreactivity for nestin showed strong expression at 33°C in DM-10 and in RA medium. A shift to 39°C resulted in a decrease of this expression of similar magnitude in both medium (Fig 4.6 (C); Fig 4.15; Table 4.8).

Mash-1 transcripts were absent at 33°C in both RA medium and in DM-10. A shift to 39°C in either growth medium resulted in an increase in expression [Fig 4.4 (C)].

BF-1 transcripts were consistently expressed at 39°C in DM-10 and at both permissive and non-permissive temperatures in RA medium. No BF-1 message was seen at 33°C in DM-10 [Fig 4.4 (D)].

At 33°C in either DM-10 or in RA medium, OR transcripts was not detected. Expression of an OR PCR product could be detected in cells cultivated at 39°C under both media conditions [Fig 4.4 (G)].

Table 4.7 Summarised results of the RT-PCR performed on RNA from OP55 cells maintained in the DM-10 medium and the RA medium (RA) experiments. The OP55 cells were maintained in culture at 33°C or at 39°C for 4, 8, 12, or 18 days. The expression of a particular marker is indicated by (+) or the absence of expression by (-). OR is RT-PCR for the olfactory receptor neuron transcripts.

Cell line	Medium	β-actin	Olf-1	Mash-1	BF-1	Pax6	GnRH	OR	Otx2
OP55 33°C	**DM-10**	+	+	-	-	-	-	-	+
OP55 39°C-4		+	+	+	+	-	-	+	+
OP55 39°C-8		+	+	+	+	-	-	+	+
OP55 39°C-12		+	+	+	+	-	-	+	+
OP55 39°C-18		+	+	+	+	-	-	+	+
OP55 33°C	**RA**	+	+	-	+	-	-	-	+
OP55 39°C-4		+	+	+	+	-	-	+	+
OP55 39°C-8		+	+	+	+	-	-	+	+
OP55 39°C-12		+	+	+	+	-	-	+	+
OP55 39°C-18		+	+	+	+	-	-	+	+

Table 4.8 Summarised results of the ICC on OP55 cells maintained in the DM-10 and RA medium (RA). The OP55 cells were maintained in culture at 33°C or at 39°C for 4, 8, 12, or 18 days. The expression of a particular marker is indicated by (+++) for very strong positive staining; (++) for weaker positive staining; (bg) for background staining and (-) for the absence of stained cells. Slides in which both positive and negative cells were present, six randomly chosen fields were counted for each time point and the percentage (%) positive cells ± SD is given instead of a symbol.

Cell line	Medium	Nestin	Vimentin	NF160	GFAP	O1	Keratin
OP55 33°C	**DM-10**	+++	-	-	-	-	++
OP55 39°C-4		+++	-	-	-	-	-
OP55 39°C-8		73.50±4.32	-	-	-	-	-
OP55 39°C-12		49.00±6.78	-	-/ bg	-	-	-/ bg
OP55 39°C-18		41.50±7.42	-	-/ bg	-/ bg	-	-/ bg
OP55 33°C	**RA**	+++	-	-	-	-/ bg	++
OP55 39°C-4		+++	-	-	-	-/ bg	++
OP55 39°C-8		83.33±7.81	-	-	-	bg	++
OP55 39°C-12		41.50±6.50	-	-	-	bg	++
OP55 39°C-18		56.33±10.75	-	-/ bg	-/ bg	-/ bg	++

4.4 DISCUSSION

Do the OP cells differentiate when shifted to the non-permissive temperature?

To determine whether the OP cell lines could differentiate, they were shifted to 39°C for a period of 18 days in the presence of the morphogen, retinoic acid. The cells were maintained for 18 days at 39°C for a number of reasons: It was important to show that the SV40 Tag was no longer expressed since it has been shown to inhibit differentiation (Bhalero *et al.* 1995). I found that after 10 days at 39°C in DM-10, the SV40 Tag was no longer detectable immunochemically and thus presumably longer active and able to induce proliferation of the OP cells. Wittemore and White (1993) showed RA-induced differentiation was a slow process that could take 1-2 weeks in culture. I wanted to grow the cells for a period of approximately a week beyond this point and maximise the time for the cells to differentiate. Previous experiments with growing the OP cells at 39°C showed that they were viable for a maximum period of 3 weeks. Beyond this time point the OP cells appeared morphologically unhealthy. This is consistent with other experiments using tissues and dissociated cultures that have shown that such cells seldom survive in culture for as long as 2-3 weeks. Most cultures are only viable for 5-7 days.

The optimal growth conditions for differentiation of the OP cells involved plating them out at a density of $1x10^4$ cells/cm^2, allowing them to settle at 33°C and to reach approximately 60-70% confluency before being shifted to 39°C. This was necessary because half of the cells usually died shortly after shifting to 39°C and, with lower starting numbers the cell density of the remainder was sub-critical for survival at 39°C.

As an alternative or additional manoeuvre for inducing differentiation, I grew the cells in N2 medium containing 10^{-6} M RA (Bhalerao *et al.* 1995; Boylan *et al.* 1993; Kaplin *et al.* 1993) in the presence of 2% FCS. The N2 medium was designed to support proliferation of neuronal cells (Bottenstein and Sato 1979). The OP cells did not grow in serum-free medium. The addition of 2% FCS was the lowest concentration of FCS that would support survival in N2 medium containing RA for a period of approximately 3 weeks.

Differentiation of the OP cell lines was analysed morphologically and phenotypically at 39°C and in comparison with cells maintained under standard growth conditions of DM-10 medium.

4.4.1 *Differentiation of the OP6 cell line*

OP6 cell lines maintained in DM-10 or RA medium at 39°C became refractile and extended processes to resemble a neuronal morphology. These extensions grew longer as the cells were maintained in culture and extended towards neighbouring cells with which they made contact to form an interconnecting network. In this respect OP6 cells resembled the Class 2 cells immortalised from olfactory epithelium of adult mice by MacDonald *et al.* (1996). The cells in Class 2 could be distinguished by three different morphologies: Some were flat, epithelial-like cells; others were round refractile cells; and yet others were bipolar cells with short processes. Morphologically, therefore, the OP6 cells maintained at 39°C displayed characteristics of olfactory receptor neurons *in vitro.* While more definitive proof is required to identify their extensions as neurites, the morphological changes that I observed were consistent with this interpretation. In this respect, therefore, it seems likely that differentiation was induced, although RA did not have significant effect on morphology. Cells cultured in the presence of RA did, however, survive beyond 18 days.

The differentiation of the OP6 cell line into several cell types with different morphology was supported by the phenotypic findings. The ICC results with nestin antibody saw the loss of expression in some cells, whereas nestin expression was retained in others. This was particularly noticeable in DM-10 medium. This should be taken into consideration when interpreting the results of the RT-PCR, since the RT-PCR represented the results of the entire population of cells and did not identify different populations within an experimental group.

Initial studies of OP6 cells showed, both by RT-PCR (Fig 3.7) and by ICC that nestin, was strongly expressed at 33°C in DM-10 and in RA medium. As the OP6 cells were shifted from 33°C to 39°C, there was a decrease in the immunoreactivity of the nestin marker. In the RA medium there was a 10-20% loss and in the DM-10 medium there was a 60-70% loss of nestin immunoreactivity (Table 4.2). Nestin-negative cells could have been OP6 cells that had started to differentiate. In the RA medium, the OP6 cells expressed nestin at 33°C and a shift to 39°C resulted in a decrease in nestin immunoreactivity. From the ICC results we see a dramatic drop in nestin protein expression in the DM-10 medium.

Some of the OP6 cells maintained at 39°C ceased to display nestin protein. If that, was, indeed indicative of differentiation the question arose: were these cells differentiating along a neuronal or a non-neuronal pathway? Antibodies to the non-neuronal markers, O1 and GFAP did not stain these

cells suggesting that the OP6 cell line was not of the non-neuronal lineage. This was supported by the lack of transcripts of Pax6 - a marker of non-neuronal cells in the olfactory system.

To identify a more mature neuronal phenotype I used antibodies to NF160. The OP6 cell line had high background staining of NF160 at 39°C in RA medium (Table 4.2). This high background staining with the antibody was also detected in the negative control slide at 39°C. This was possibly due to the cells not being very healthy after 1-2 weeks at 39°C. It has been shown that damaged or dying cells do tend to stain non-specifically (Bourne, Dako handbook). Furthermore, while NF160 is expressed in the olfactory system *in vitro* (Chuah *et al.* 1991), *in vivo* studies have shown that the olfactory system retains juvenile markers and that less than 0.1% of the cells express the neurofilament markers (Schwob *et al.* 1986).

The results thus far suggest that the OP6 cell line differentiated along a neuronal pathway. To document more convincingly I used RT-PCR to look for transcripts of the proliferating and differentiating markers BF-1, Mash-1 and Otx2.

Mash-1 promotes determination of an already committed precursor cell rather than an uncommitted stem cell (Bang & Goulding 1996; Simpson 1995; Sommer *et al.* 1995). The OP6 cell line lacked expression of the Mash-1 marker at 39°C. The OP6 cells at 39°C could thus represent an uncommitted stem cell or it is possible that the population of cells that had differentiated was small and the expression of Mash-1 was thus too low to be detected.

BF-1 has been shown to play a role in proliferation and in timing of differentiation of precursor cells into a more differentiated neuronal phenotype (Tao & Lai 1992). The expression of BF-1 at 39°C was faint and it is, therefore, possible that only a subpopulation of the cells expressed this marker. The BF-1 positive cells may have belonged to the same population of undifferentiated cells that continued to express nestin. As I have pointed out earlier, the RT-PCR results reflect transcript density in the entire cell population; they do not distinguish between cells that are positive and those that were negative for a particular marker. The expression of BF-1 may thus have represented a sub-population of proliferating cells rather than the entire population.

Further support for the differentiation of the OP6 cells into a neuronal phenotype was the increase in expression of the Otx2. This marker has been shown to be expressed in the olfactory receptor neurons and their precursors, the INPs (Calof *et al.* 1996). In the OP6 cell line the expression for the Otx2 marker was strongest in the RA medium when the cells were maintained at 39°C. The

expression for Otx2 increased as the cells were maintained at 39°C in RA medium. This result is in contrast to *in vivo* studies, where RA applied exogenously resulted in a decrease in expression of Otx2.

The RT-PCR results provided strong evidence that a subpopulation of the OP6 cells were induced to differentiate along a neuronal pathway. The expression of the olfactory receptor transcripts suggests that the OP6 cell line contained olfactory receptor neuronal precursors that, when shifted to 39°C, differentiated into neurons.

4.4.2 *Differentiation of the OP27 cell line*

In DM-10 at 39°C, OP27 cells became refractile and extended long processes, some of which were unipolar and others multipolar. These resembled, in fact, the cultured olfactory cells, described by Pixley & Pun (1990), that responded to odours and that were bipolar, with some of their processes branched. In this respect they differed from olfactory neurons *in vivo* which are bipolar, with one axon and dendrite both of which are unbranched.

Once the OP27 cells were shifted from 33°C to 39°C in the RA medium, they became large, flat cells that grew larger as they were maintained at 39°C. Only a small percentage (approximately 10% of the total cell population) became phase bright and developed extensions that could be said to be characteristic of neuronal cells. OP27 represented a similar morphology to Class 2 cells by MacDonald *et al.* (1996) as described for the OP6 cells. The possibility that the OP27 cells or the majority of them did not differentiate in response to RA and a temperature shift is supported by the continuous expression of nestin immunoreactivity when the cells were shifted to 39°C, by the continued expression of the proliferating marker BF-1, and by the lack of expression of the differentiation marker, Mash-1.

As the OP27 cells were maintained longer at 39°C in the RA medium, a decrease in expression of the BF-1 marker was detected by RT-PCR. This could imply that, if differentiation did take place, it happened after a delay at 39°C and that these cells might require a longer period at 39°C in order for the differentiated phenotype to become more obvious.

The OP27 cell line did not, however, display a complete lack of differentiation as a change in expression of the Pax6 marker was shown by RT-PCR. Pax6 expression has been detected in the horizontal basal cell population and in the non-neuronal lineage of the olfactory epithelium (Davis

& Reed 1996). The OP27 cell line could thus represent a horizontal basal cell population and this was supported by the flat morphology of the cells at 39°C and the continuous expression of the horizontal basal cell marker, keratin. Pax6 is a marker of the non-neuronal cell lineage of the olfactory epithelium (Davis & Reed 1996), however, the OP27 cell line did not differentiate to express the more mature non-neuronal markers of GFAP or O1, as determined by ICC.

The markers used to detect differentiation of the OP27 cell line did not show a dramatic change in expression with RA and/or a shift to 39°C. However, the change in expression of the Pax6 marker as well as the increase in expression of the olfactory receptor transcripts when the OP27 cell line was shifted to 39°C indicated that differentiation of the OP27 cells had been induced. The differentiation of the OP27 cell line into olfactory receptor neurons was further supported by the expression of the differentiating olfactory neuronal marker, Otx2. Otx2 has been shown to be expressed in the olfactory receptor neurons and their precursors (Calof *et al.* 1996). The expressions of the flat morphology, Olf-1, Pax6, keratin and the olfactory receptor transcripts provided evidence that the OP27 cell line belonged to the horizontal basal cell population or a subpopulation thereof. This supports the evidence that the horizontal basal cells, or a subpopulation thereof, eventually give rise to olfactory receptor neurons. This lineage for the olfactory receptor neurons from the horizontal basal cells is supported by some researchers (Monti Grazaidei & Grazaidei 1979; Satoh & Takeuchi 1995; Schwartz Levey *et al.* 1991).

4.4.3 *Differentiation of the OP47 cell line*

The OP47 cells grown in DM-10 at 39°C became refractile and extended unipolar or bipolar processes that varied in length. Positioned on top of a layer of large, flat cells, these refractile cells resembled the *in vitro* olfactory receptor neurons described by Pixley and Pun (1990). Although the major cell type at 39°C had a flat morphology, the longer the OP47 cell line was maintained at 39°C the more a second cell type became prominent. This consisted of phase-bright cells with long processes that were typical of neuronal cells and that connected with other phase-bright cells in culture. As neuronal cell morphology developed, so nestin immunoreactivity increased. Initially, at 33°C, the OP47 cell line was negative for nestin but, as the cells were maintained at 39°C expression of this multipotential stem cell marker increased. Further evidence of OP47 cell differentiation at 39°C was provided by the expression of the differentiation marker, Mash-1 and the transcript for olfactory receptors. Mash-1 was detected once the cells were shifted to 39°C in both media, but its expression started at an earlier stage in the RA medium. The expression of OR was higher in the presence of RA than in DM-10 at 39°C

At the time which I stopped observing OP47 cells at 39°C, the proliferation marker, BF-1, was still expressed, suggesting that a subpopulation of the cells had not differentiated.

The fact that the OP47 cells were negative for nestin at 33°C and only became positive at 39°C, raised the interesting possibility that these were, infact, early stem cells that had been immortalised **before** nestin expression. As very early stem cells one would have expected them, once encouraged to differentiate, to divide asymmetrically giving one daughter cell that advanced to the next maturation stage and expressed nestin while the other daughter cell remained as a stem cell and continued to express BF-1.

This possibility was supported by the observation that I made and, to some extent, by the fact that OP47 cell cultures remained healthy at 39°C and maintained their numbers at a constant level - the cellular kinetics one would expect from a mixed population of terminally differentiated cells and a smaller population of sustained stem cells.

The failure of 39°C cultures to incorporate BrdU (section 3.3.6) does, to some extent, argue against this possibility but does not exclude it, since low stem cell numbers and long doubling times might have made BrdU incorporation insufficiently sensitive to detect proliferation.

4.4.4 *Differentiation of the OP55 cells*

Unlike the other cell lines, OP55 cells when shifted to 39°C did not become refractile or develop processes. Instead they remained rounded with some being large and flat while others were smaller and compact.

The compact cells fitted tightly and closely together to form organised structures resembling epithelial islands. Keratin expression and the morphological resemblance that they bore to the horizontal basal cell described by Suzuki and Takeda (1993) and the Class I cells of MacDonald *et al.* (1996) suggest strongly that the OP55 cells were horizontal basal cell precursors.

Further evidence for the induction of differentiation in OP55 cells was provided by the down-regulation of the nestin marker (Table 4.8), the up-regulation of the Mash-1 marker and olfactory receptor transcripts (Table 4.7).

The RT-PCR results with the BF-1 primers showed continued expression, at 39°C, both in the RA medium and in the DM-10 medium indicating that a subpopulation of OP55 cells remained undifferentiated. Heterologous expression of nestin ICC further supported this idea.

4.4.5 *Dedifferentiation of the OP cell lines at 33°C*

During the course of these studies I noticed that early passage (i.e. < 3-4) of OP cells maintained at 33°C expressed markers (such as GnRH, nestin, Pax6 and BF-1) that were lost with repeated passaging. This apparent "de-differentiation" (or selection for clones that had lost marker expression) has been reported by others who have used SV40 for immortalisation (Almazan & McKay 1992; McLean 1993; Prince *et al.* 1998; S. Prince, personal communication) and emphasise the need for experiments to be performed on a restricted passage number - preferably before the 15th passage.

Markers (with the exception of GnRH) that were "lost" at 33°C were "regained" when the cells were shifted to 39°C, supporting the concept of "de-differentiation" as being responsible for the phenomenon.

4.4.6 *Differentiation in RA medium compared to the in DM-10 medium*

I did not observe any striking additive or synergistic effects between RA and the temperature shift. There were occasions when I felt that, perhaps, RA forced the growth of one cell type in culture whereas, in DM-10 at 39°C, both cell types were equally expressed. But these impressions were not sufficiently consistent or obvious for me to document them with confidence.

Unlike the reports on the EC cell lines, or *in vivo* evidence suggesting that RA is a potent inducer of neuronal differentiation, there was no significant difference between cells cultured in DM-10 and in RA at 39°C. There were small effects on different markers, but no clear overall trend. All four cell lines showed expression of OR (most mature olfactory neuronal marker) in DM-10 and RA medium.

4.4.7 *Expression of the olfactory receptor transcript*

All the four OP cell lines expressed the transcript for the olfactory receptors, once shifted to 39°C, either in the DM-10 medium or the RA medium. This provided strong evidence for differentiation of the OP cell lines from a precursor cell to an immature olfactory receptor expressing neuron. The nature of this expression is explored in more detail in chapter 5.

4.4.8 *Expression of the other markers*

Although RT-PCR is not an accurate quantitative procedure, some overall trends were observed. Several different patterns of expression emerged for the different markers used in this study. Interestingly Olf-1 expression, like β-actin, remained relatively constant under all growth conditions. Olf-1 is a transcription factor which is expressed in the olfactory epithelium during embryogenesis and in the mature animal. It is believed to play a critical role in regulating genes that function in odorant detection and neuronal turnover. The continuous expression of Olf-1 in all 4 cell lines confirms the olfactory origin of these cells and their differentiation along the olfactory neuronal pathway rather than the olfactory sustentacular pathway. Olf-1 was only detected in olfactory basal cells, immature and mature olfactory receptor neurons but not in olfactory sustentacular cells (Davis & Reed 1996).

BF-1, a transcription factor, is expressed in proliferating progenitor cells as early as embryonic day 8.5 and has a peak period at E14-17, coincident with the major period of neuronal proliferation. It was is up-regulated at 39°C in DM-10 and continuously expressed in all cell lines in RA medium. This expression of BF-1 strongly suggest that a population of cells continues to express this immature, proliferating marker and not all cells differentiate once shifted to 39°C.

Mash-1, a marker expressed in olfactory basal cells and is thought to be involved in proliferation and initiation of differentiation to postmitotic cells. The up-regulation of Mash-1 in the OP47 and OP55 cells at 39°C supports the BF-1 results which suggest that a subpopulation of cells continues to remain as basal cells which do not differentiate. Alternatively, Mash-1 expression may imply initiation of differentiation once the cells are shifted to 39°C.

The onset of olfactory receptor expression were found between E12 and E14 by In Situ hybridisation (Strotman *et al.* 1995) and serve as a marker for differentiation of basal cells to immature and mature neurons. All four cell lines started to express the transcript once shifted to

39°C in DM-10 or in RA medium at 33°C and at 39°C. OP6 cells expressed higher levels of this transcript in DM-10 while OP47 expressed higher levels in RA medium. OP27 and OP55 expressed the transcript in both media conditions. This would suggest that for OP6, a shift to 39°C was sufficient to induce differentiation while for the OP47 cells a shift to 39°C and the addition of RA was required . For the OP27 and the OP55 cells, RA had no additive effect on the induction of the olfactory receptor transcripts.

Otx2 is broadly expressed early during embryogenesis. Explant cultures (Calof *et al.* 1996) restricted the expression of Otx2 to olfactory progenitor cells which lack neurites while *in vivo* studies were only able to detect Otx2 in postmitotic neurons. All four cell lines showed different expression for Otx2. OP27 and OP55 cells continued to express it while in OP6 cells Otx2 was up-regulated at 39°C and in OP47 cells Otx2 was down-regulated at 39°C. *In vivo* studies (Simeone *et al.* 1995) showed that RA results in the down-regulation of Otx2 marker. The OP47 cells confirmed these *in vivo* results but the opposite is seen in the OP6 cells and RA had no effect on the OP27 and the OP55 cell lines.

Nestin is marker of proliferating stem cells in the CNS. As cells differentiate, they loose nestin expression and this was clearly seen for OP6 and OP55 cells, where nestin immunoreactivity was lost with a shift from 33°C to 39°C. For OP6 cells the loss was greater in DM-10 than in RA medium, suggesting that more cells differentiated in the DM-10 medium. This was further supported by the greater expression of olfactory receptor transcripts in DM-10 medium compared to RA medium. The OP27 cells continued to express nestin. Perhaps the time loss of nestin expression was longer than the experimental time course. OP47 cells lacked nestin at 33°C and expressed it once shifted to 39°C. This suggests that OP47 cells are stem cells that were immortalised prior to the expression of nestin.

The ICC with nestin confirms the presence of more than one population of cells at 39°C. A population which continues to express nestin and remains as a basal cell population which retains the ability to re-enter the cell cycle at the permissive temperature. The second population of cells loses nestin expression and differentiates into immature olfactory receptor - expressing neurons.

Chapter 5

Cloning and sequencing of olfactory and taste receptors expressed by the OP cell lines at 39°C

5.1 Summary

The OP cell lines are temperature sensitive cell lines which grow indefinitely at 33°C but once shifted to the non-permissive temperature, stop proliferating and start to differentiate. Degenerate primers designed from conserved sequences in the rat olfactory receptor gene family were used to determine whether the OP cells expressed these transcripts once shifted to 39°C. All the cell lines amplified an expected size PCR product to olfactory receptor transcripts at 39°C. Sequence analysis of the PCR products revealed that the degenerate primers amplified transcripts to a single type of olfactory receptors in the OP27 and the OP55 cell lines while they amplified transcripts to taste receptors in the OP6 and the OP47 cell lines. Taste receptors are G-protein coupled receptors with high homology to the olfactory receptor gene family. The OP6 and the OP47 cell lines expressed the same receptor which had highest homology to known taste receptor Gus58-rat (78%). The OP27 cell line expressed a transcript with highest homology to the olfactory receptor M65 (98%) and the OP55 cell line expressed a transcript with highest homology to the olfactory receptor Olf3-rat (91%). The receptors cloned from the OP6, OP47 and OP55 cell lines were not 100% identical to known receptors and could represent new members of the olfactory and the taste receptor gene family.

5.2 Introduction

The four olfactory cell lines OP6, OP27, OP47 and OP55 were immortalised with the temperature sensitive SV40 Tag. In order to induce differentiation they were shifted to 39°C and/or exposed to RA. By the criteria of morphology, mRNA transcription (by RT-PCR) and protein expression (by ICC) these cells were shown to differentiate from precursor cells to those with a more mature neuronal phenotype. I considered the expression of olfactory receptors in these cell lines as the most compelling evidence of induced differentiation. In this chapter I describe experiments designed to confirm these findings and to characterise the receptors in more detail.

5.3 Experimental protocol

Cultures of the four cell lines OP6, OP27, OP47 and OP55 where shifted to 39°C, in the presence or absence of RA, and at various time intervals, they were analysed by RT-PCR for olfactory receptor transcripts. The reverse transcribed cDNA product was amplified using Taq DNA polymerase and the primers OR3 (5' AGATCTAGATGGCITAT(C)GAT(C)C(A)GITA (T)T(C)GTIGC 3') and ORC (5' GCTCTAGATAA(GT)ATA(G)AAIGGA(G)TTIAA(G)CA T 3') to generated cDNA fragments with 'A' overhangs (Hu 1993; Liu 1996) which could be cloned directly into the pGEM - T vector. This 'TA cloning' strategy has been well described and is conveniently available in kit form (Promega).

The final choice for inducing conditions with each cell line was made on the basis of those conditions that yielded the strongest band on the first round of PCR amplification [Fig 4.4 (G)]. These were as follows:

1] OP6 grown in DM-10 - 39°C for 12 days

2] OP27 grown in RA medium - 39°C for 8 days

3] OP47 grown in RA medium - 39°C for 8 days

4] OP55 grown in RA medium - 39°C for 8 days

The PCR products were electrophoresed in a low melting point agarose gel, excised, purified and ligated into the pGEM-T vector overnight and transformed into competent *E. coli*. Positive transformants were identified by 'mini-preps' of DNA and digested with Pst1 and Sph1, which cut in the multiple cloning site of the vector, or by colony PCR (section 2.5.5). Transformants yielding a product of 550 bp were chosen for further analysis. Purified DNA was prepared from two positive clones representing each cell line and sequenced on an automated DNA sequencer.

For restriction enzyme analysis to test for singularity of the expressed putative receptors, colony PCR was performed on transformants for each cell line. Approximately 20 positive clones were chosen and digested. The protocols and the enzymes used for this latter analysis are detailed in the results section (Table 5.4).

5.4 Results

5.4.1 *Sequence homology of clones in different cell lines*

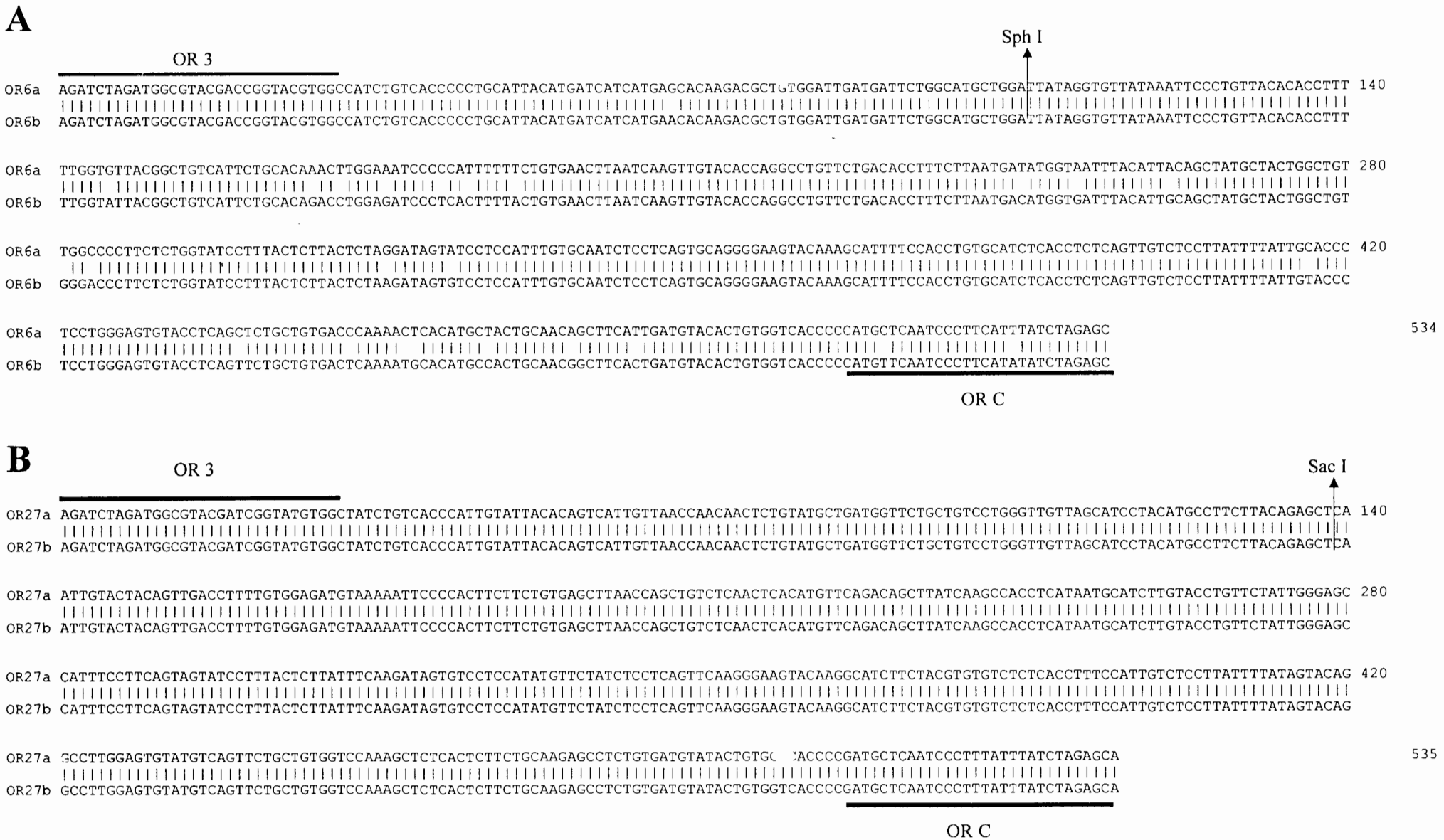
Sequences were obtained for RT-PCR products from duplicates of the four cell lines. These I refer to as OR6-a and -b; OR27-a and -b; OR47-a and -b and OR55-a and -b. All of the sequences contained the forward and the reverse primers flanking open reading frames (ORF's) (i.e. sequences without stop codons) of approximately 530 bp.

Compared to each other, the duplicate sequences for the three cell lines (OP6, OP27 and OP47) were virtually identical [Fig 5.1 (A-C)] with such differences as there were (25 nt/536 nt for OP6a vs. OP6b; 8 nt/534 nt for OP47a vs. OP47b; 0/535 nt for OP27a vs. OP27b) explicable by the infidelity of PCR. The discrepancies observed are not significant evidence of more than one receptor per cell line.

The sequences of the RT-PCR products for OP6 and OP47 were very nearly identical. The differences observed were almost entirely due to the bases in OP6b that were not homologous with these in OP6a or OP47.

OP6 and OP47 were, therefore, probably derived from the same ancestral clone or they represent two different clones that express the same receptor. OP55-a and -b differed extensively [Fig 5.1(D)]. The degree of homology at the nucleotide level between the ORF's for the different clones is summarised in Table 5.1

Fig 5.1 The nucleotide sequence encoded by the two cDNA clones from each of the OP6 (A), OP27 (B), OP47 (C) and OP55 (D) cell lines. Conserved nucleotides are indicated by a |. Overlines indicate the forward (OR F3) and the reverse (OR RC) primers. The restriction sites for the restriction analysis is indicated with an arrow; Sph I for OP6 and OP47; Sac I for OP27; Hind III for OP55 and Bgl II for clusterin (OP55b).


A
OR 3
Sph I
OR6a AGATCTAGATGGCGTACGACCGGTACGTGGCCATCTGTCACCCCCTGCATTACATGATCATCATGAGCACAAGACGCTGTGGATTGATGATTCTGGCATGCTGGATTATAGGTGTTATAAATTCCCTGTTACACACCTTT 140
OR6b AGATCTAGATGGCGTACGACCGGTACGTGGCCATCTGTCACCCCCTGCATTACATGATCATCATGAACACAAGACGCTGTGGATTGATGATTCTGGCATGCTGGATTATAGGTGTTATAAATTCCCTGTTACACACCTTT
OR6a TTGGTGTTACGGCTGTCATTCTGCACAAACTTGGAAATCCCCCATTTTTTCTGTGAACTTAATCAAGTTGTACACCAGGCCTGTTCTGACACCTTTCTTAATGATATGGTAATTTACATTACAGCTATGCTACTGGCTGT 280
OR6b TTGGTATTACGGCTGTCATTCTGCACAGACCTGGAGATCCCTCACTTTTACTGTGAACTTAATCAAGTTGTACACCAGGCCTGTTCTGACACCTTTCTTAATGACATGGTGATTTACATTGCAGCTATGCTACTGGCTGT
OR6a TGGCCCCTTCTCTGGTATCCTTTACTCTTACTCTAGGATAGTATCCTCCATTTGTGCAATCTCCTCAGTGCAGGGGAAGTACAAAGCATTTTCCACCTGTGCATCTCACCTCTCAGTTGTCTCCTTATTTTATTGCACCC 420
OR6b GGGACCCTTCTCTGGTATCCTTTACTCTTACTCTAAGATAGTGTCCTCCATTTGTGCAATCTCCTCAGTGCAGGGGAAGTACAAAGCATTTTCCACCTGTGCATCTCACCTCTCAGTTGTCTCCTTATTTTATTGTACCC
OR6a TCCTGGGAGTGTACCTCAGCTCTGCTGTGACCCAAAACTCACATGCTACTGCAACAGCTTCATTGATGTACACTGTGGTCACCCCCATGCTCAATCCCTTCATTTATCTAGAGC 534
OR6b TCCTGGGAGTGTACCTCAGTTCTGCTGTGACTCAAAATGCACATGCCACTGCAACGGCTTCACTGATGTACACTGTGGTCACCCCCATGTTCAATCCCTTCATATATCTAGAGC
OR C
B
OR 3
Sac I
OR27a AGATCTAGATGGCGTACGATCGGTATGTGGCTATCTGTCACCCATTGTATTACACAGTCATTGTTAACCAACAACTCTGTATGCTGATGGTTCTGCTGTCCTGGGTTGTTAGCATCCTACATGCCTTCTTACAGAGCTCA 140
OR27b AGATCTAGATGGCGTACGATCGGTATGTGGCTATCTGTCACCCATTGTATTACACAGTCATTGTTAACCAACAACTCTGTATGCTGATGGTTCTGCTGTCCTGGGTTGTTAGCATCCTACATGCCTTCTTACAGAGCTCA
OR27a ATTGTACTACAGTTGACCTTTTGTGGAGATGTAAAAATTCCCCACTTCTTCTGTGAGCTTAACCAGCTGTCTCAACTCACATGTTCAGACAGCTTATCAAGCCACCTCATAATGCATCTTGTACCTGTTCTATTGGGAGC 280
OR27b ATTGTACTACAGTTGACCTTTTGTGGAGATGTAAAAATTCCCCACTTCTTCTGTGAGCTTAACCAGCTGTCTCAACTCACATGTTCAGACAGCTTATCAAGCCACCTCATAATGCATCTTGTACCTGTTCTATTGGGAGC
OR27a CATTTCCTTCAGTAGTATCCTTTACTCTTATTTCAAGATAGTGTCCTCCATATGTTCTATCTCCTCAGTTCAAGGGAAGTACAAGGCATCTTCTACGTGTGTCTCTCACCTTTCCATTGTCTCCTTATTTTATAGTACAG 420
OR27b CATTTCCTTCAGTAGTATCCTTTACTCTTATTTCAAGATAGTGTCCTCCATATGTTCTATCTCCTCAGTTCAAGGGAAGTACAAGGCATCTTCTACGTGTGTCTCTCACCTTTCCATTGTCTCCTTATTTTATAGTACAG
OR27a GCCTTGGAGTGTATGTCAGTTCTGCTGTGGTCCAAAGCTCTCACTCTTCTGCAAGAGCCTCTGTGATGTATACTGTGC ACCCCGATGCTCAATCCCTTTATTTATCTAGAGCA 535
OR27b GCCTTGGAGTGTATGTCAGTTCTGCTGTGGTCCAAAGCTCTCACTCTTCTGCAAGAGCCTCTGTGATGTATACTGTGGTCACCCCGATGCTCAATCCCTTTATTTATCTAGAGCA
OR C

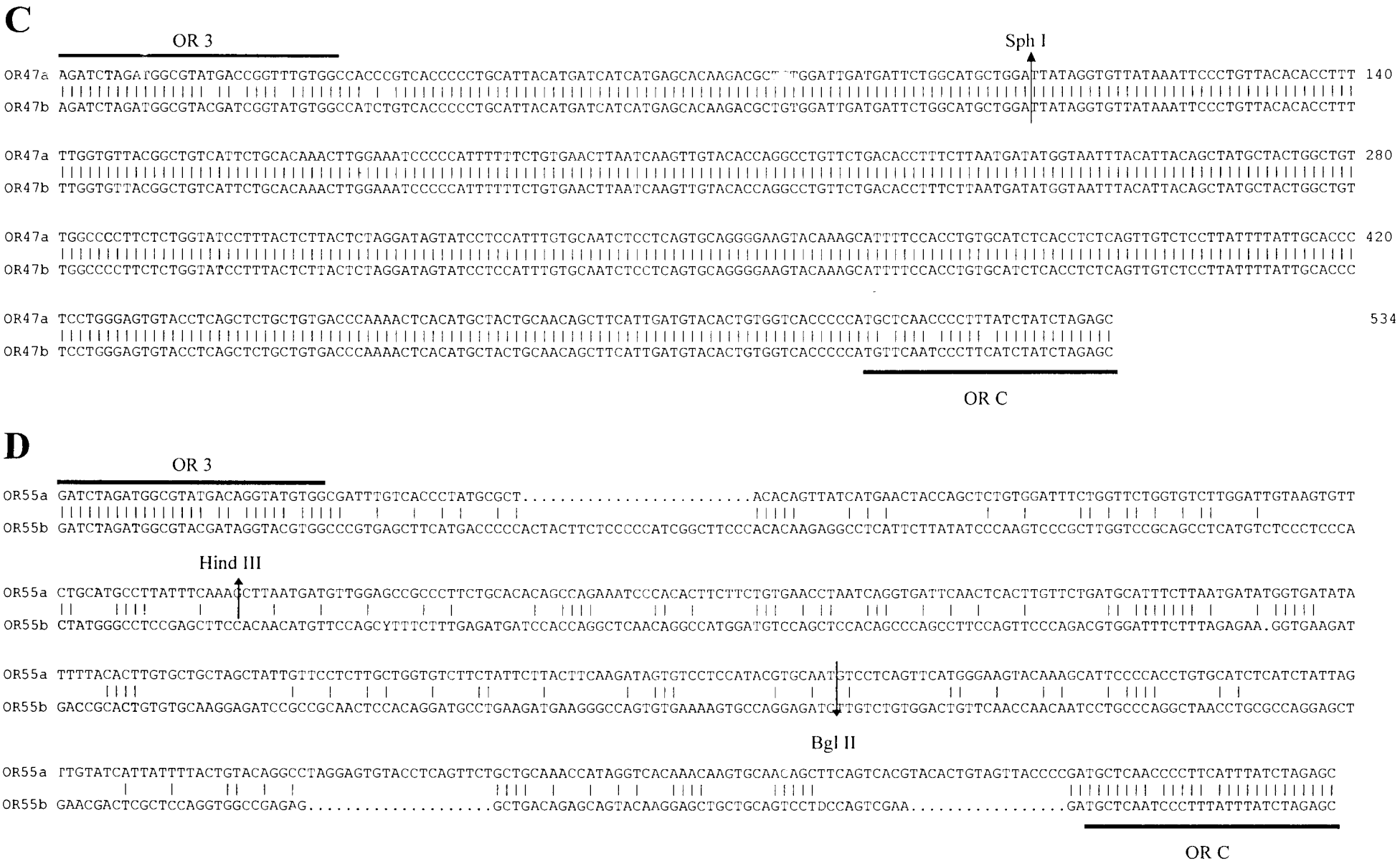

C
OR 3
Sph I
OR47a AGATCTAGATGGCGTATGACCGGTTTGTGGCCACCCGTCACCCCCTGCATTACATGATCATCATGAGCACAAGACGCTGTGGATTGATGATTCTGGCATGCTGGATTATAGGTGTTATAAATTCCCTGTTACACACCTTT 140
OR47b AGATCTAGATGGCGTACGATCGGTATGTGGCCATCTGTCACCCCCTGCATTACATGATCATCATGAGCACAAGACGCTGTGGATTGATGATTCTGGCATGCTGGATTATAGGTGTTATAAATTCCCTGTTACACACCTTT
OR47a TTGGTGTTACGGCTGTCATTCTGCACAAACTTGGAAATCCCCCATTTTTTCTGTGAACTTAATCAAGTTGTACACCAGGCCTGTTCTGACACCTTTCTTAATGATATGGTAATTTACATTACAGCTATGCTACTGGCTGT 280
OR47b TTGGTGTTACGGCTGTCATTCTGCACAAACTTGGAAATCCCCCATTTTTTCTGTGAACTTAATCAAGTTGTACACCAGGCCTGTTCTGACACCTTTCTTAATGATATGGTAATTTACATTACAGCTATGCTACTGGCTGT
OR47a TGGCCCCTTCTCTGGTATCCTTTACTCTTACTCTAGGATAGTATCCTCCATTTGTGCAATCTCCTCAGTGCAGGGGAAGTACAAAGCATTTTCCACCTGTGCATCTCACCTCTCAGTTGTCTCCTTATTTTATTGCACCC 420
OR47b TGGCCCCTTCTCTGGTATCCTTTACTCTTACTCTAGGATAGTATCCTCCATTTGTGCAATCTCCTCAGTGCAGGGGAAGTACAAAGCATTTTCCACCTGTGCATCTCACCTCTCAGTTGTCTCCTTATTTTATTGCACCC
OR47a TCCTGGGAGTGTACCTCAGCTCTGCTGTGACCCAAAACTCACATGCTACTGCAACAGCTTCATTGATGTACACTGTGGTCACCCCCATGCTCAACCCCTTTATCTATCTAGAGC 534
OR47b TCCTGGGAGTGTACCTCAGCTCTGCTGTGACCCAAAACTCACATGCTACTGCAACAGCTTCATTGATGTACACTGTGGTCACCCCCATGTTCAATCCCTTCATCTATCTAGAGC
OR C
D
OR 3
OR55a GATCTAGATGGCGTATGACAGGTATGTGGCGATTTGTCACCCTATGCGCT.....ACACAGTTATCATGAACTACCAGCTCTGTGGATTTCTGGTTCTGGTGTCTTGGATTGTAAGTGTT
OR55b GATCTAGATGGCGTACGATAGGTACGTGGCCCGTGAGCTTCATGACCCCCACTACTTCTCCCCCATCGGCTTCCCACACAAGAGGCCTCATTCTTATATCCCAAGTCCCGCTTGGTCCGCAGCCTCATGTCTCCCTCCCA
Hind III
OR55a CTGCATGCCTTATTTCAAAGCTTAATGATGTTGGAGCCGCCCTTCTGCACACAGCCAGAAATCCCACACTTCTTCTGTGAACCTAATCAGGTGATTCAACTCACTTGTTCTGATGCATTTCTTAATGATATGGTGATATA
OR55b CTATGGGCCTCCGAGCTTCCACAACATGTTCCAGCYTTTCTTTGAGATGATCCACCAGGCTCAACAGGCCATGGATGTCCAGCTCCACAGCCCAGCCTTCCAGTTCCCAGACGTGGATTTCTTTAGAGAA.GGTGAAGAT
OR55a TTTTACACTTGTGCTGCTAGCTATTGTTCCTCTTGCTGGTGTCTTCTATTCTTACTTCAAGATAGTGTCCTCCATACGTGCAATGTCCTCAGTTCATGGGAAGTACAAAGCATTCCCCACCTGTGCATCTCATCTATTAG
OR55b GACCGCACTGTGTGCAAGGAGATCCGCCGCAACTCCACAGGATGCCTGAAGATGAAGGGCCAGTGTGAAAAGTGCCAGGAGATGTTGTCTGTGGACTGTTCAACCAACAATCCTGCCCAGGCTAACCTGCGCCAGGAGCT
Bgl II
OR55a TTGTATCATTATTTTACTGTACAGGCCTAGGAGTGTACCTCAGTTCTGCTGCAAACCATAGGTCACAAACAAGTGCAACAGCTTCAGTCACGTACACTGTAGTTACCCCGATGCTCAACCCCTTCATTTATCTAGAGC
OR55b GAACGACTCGCTCCAGGTGGCCGAGAG.....GCTGACAGAGCAGTACAAGGAGCTGCTGCAGTCCTDCCAGTCGAA.....GATGCTCAATCCCTTTATTTATCTAGAGC
OR C

Table 5.1 *Homology of the clones from each cell line at the nucleotide level*

Clones	OR6a	OR6b	OR27a	OR27b	OR47a	OR47b	OR55a	OR55b
OR6a	-	95%	73%	73%	98%	98%	74%	40%
OR6b		-	71%	71%	94%	95%	75%	34%
OR27a			-	100%	70%	71%	71%	35%
OR27b				-	70%	71%	71%	35%
OR47a					-	98%	75%	39%
OR47b						-	75%	39%
OR55a							-	37%
OR55b								-

Comparison of the primary amino acid sequences deduced from the nucleotide ORF are compared in Table 5.2

Table 5.2 *Homology of the clones from each cell line at the amino acid level*

Clones	OR6a	OR6b	OR27a	OR27b	OR47a	OR47b	OR55a	OR55b
OR6a	-	96%	61%	60%	99%	98%	65%	12%
OR6b		-	61%	60%	96%	94%	64%	12%
OR27a			-	100%	59%	58%	62%	9%
OR27b				-	59%	58%	62%	9%
OR47a					-	97%	63%	12%
OR47b						-	62%	12%
OR55a							-	9%
OR55b								-

5.4.2 *Hydrophathy profiles*

The primers for the RT-PCR experiments were chosen to flank sequences encoding the C-terminal region of the TM3 and the C-terminal region of TM7. One would, therefore, have expected the deduced peptides to contain the hydrophobic domains corresponding to TM4, TM5, TM6 and TM7. Hydrophathy profiles, using the method of Kyte and Doolittle [Fig 5.2 (A-C)] showed that, for the transcripts expressed by OR6/OR47, OR27 and OR55a, this was indeed the case. OR55b did not show this pattern [Fig 5.2 (D)].

5.4.3 *Alignment of clones from the OP cell lines to known sequences*

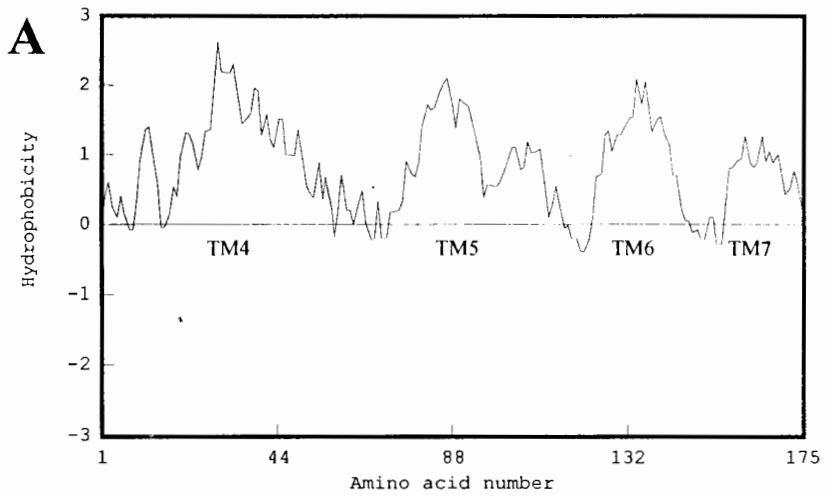
When the sequences obtained were compared to the known sequences in the Genbank and the olfactory receptor databases, the following similarities were observed:

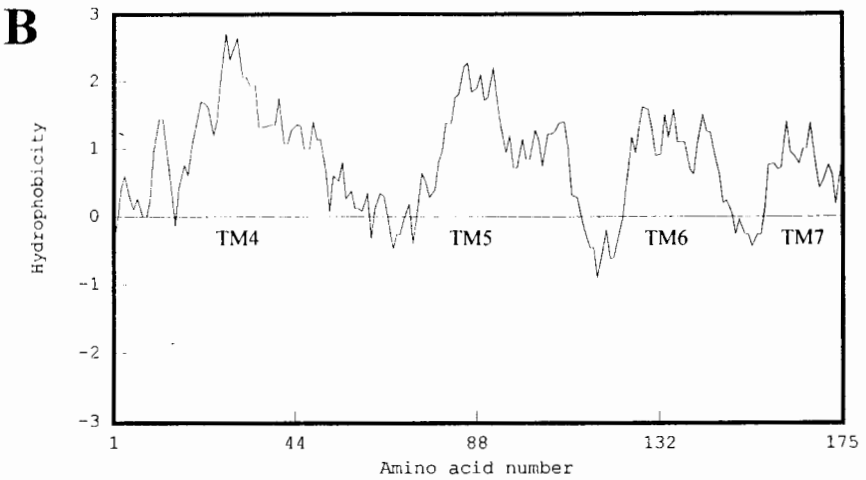
- Clones from the OP6 and the OP47 cell line showed greatest homology to the rat gustatory receptor Gus58-rat clone number pte58 (Accession number P35899) (Abe *et al.* 1993a) with a homology of 78% (Table 5.3).
- Clones from the OP27 cell line showed greatest homology to the rat olfactory receptor protein Olf2-rat clone number f12 (accession number p23268) (Buck & Axel 1991) with a homology of 88% (Table 5.3).
- The OR55a clone showed greatest homology to the rat olfactory receptor protein Olf3-rat clone f3 (accession number p23265) (Buck & Axel 1991) with a homology of 91% (Table 5.3).
- The OR55b clone showed greatest homology to the mouse clusterin precursor (accession number Q06890) (French *et al.* 1993; Jordan-Starck *et al.* 1994; Lee *et al.* 1993) with a homology of 95% (Table 5.3).

The clones OR6a, OR27a and OR55a were submitted to the Genbank database. Subsequently Peter Mombaert (from the Rockefeller University in New York) contacted my supervisor confirming homology between his independently isolated olfactory receptor clone, M65 and our OR27a clone. Furthermore, he found homology between another of his clones and our OR6a clone (Unpublished data). The olfactory clone M65 is a genomic clone isolated with a probe for the M64 receptor from Linda Buck's group, and a comparison is shown in Fig 5.3. Their clone (homologous to OR6a) was cloned by RT-PCR from the olfactory bulb that surrounds a highlighted glomerulus in their genetically mutated mice (Chapter 1 section 1.2.5). The sequence for this clone is not available.

This confirms that the OP27a clone represents an olfactory receptor which is identical to clone M65, while OP6a shows highest homology to the clone from Peter Mombaert and second greatest homology to taste receptors (Genbank).

Fig 5.2 Hydropathy profiles of clones OR6/OR47 (A), OR27 (B), OR55a (C) and OR55b (D). Plots were generated according to Kyte and Doolittle. The left vertical axis indicates the hydropathic index; the horizontal axis represents amino acid position. The major hydrophobic regions are indicated for OR6/OR47, OR27 and OR55a but OR55b had no hydrophobic regions. These hydrophobic regions could represent putative transmembrane regions (TM3 - TM7) which are the regions amplified during RT-PCR.


A
Hydrophobicity
3
2
1
0
-1
-2
-3
TM4
TM5
TM6
TM7
1
44
88
132
175
Amino acid number


B
Hydrophobicity
3
2
1
0
-1
-2
-3
TM4
TM5
TM6
TM7
1
44
88
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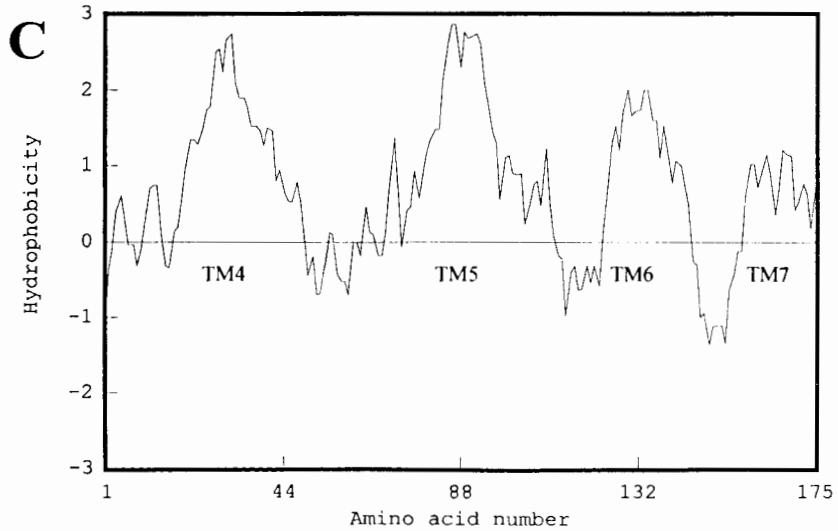
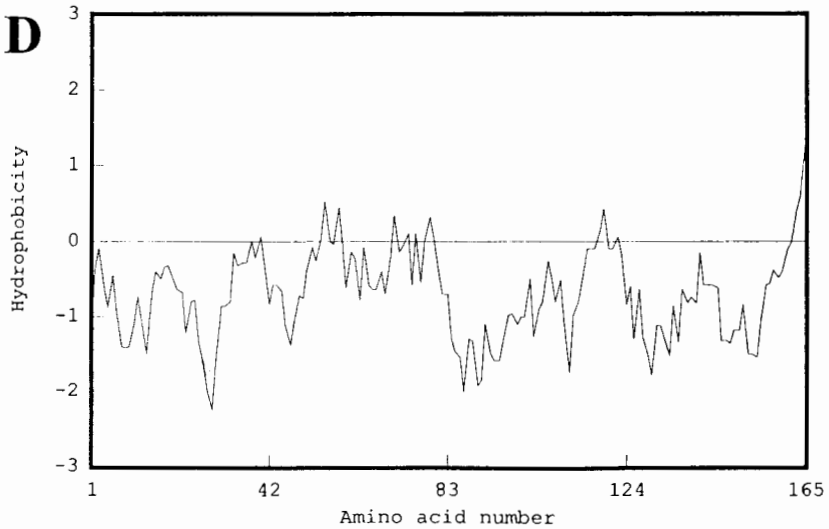
175
Amino acid number


C
Hydrophobicity
3
2
1
0
-1
-2
-3
TM4
TM5
TM6
TM7
1
44
88
132
175
Amino acid number


D
Hydrophobicity
3
2
1
0
-1
-2
-3
1
42
83
124
165
Amino acid number

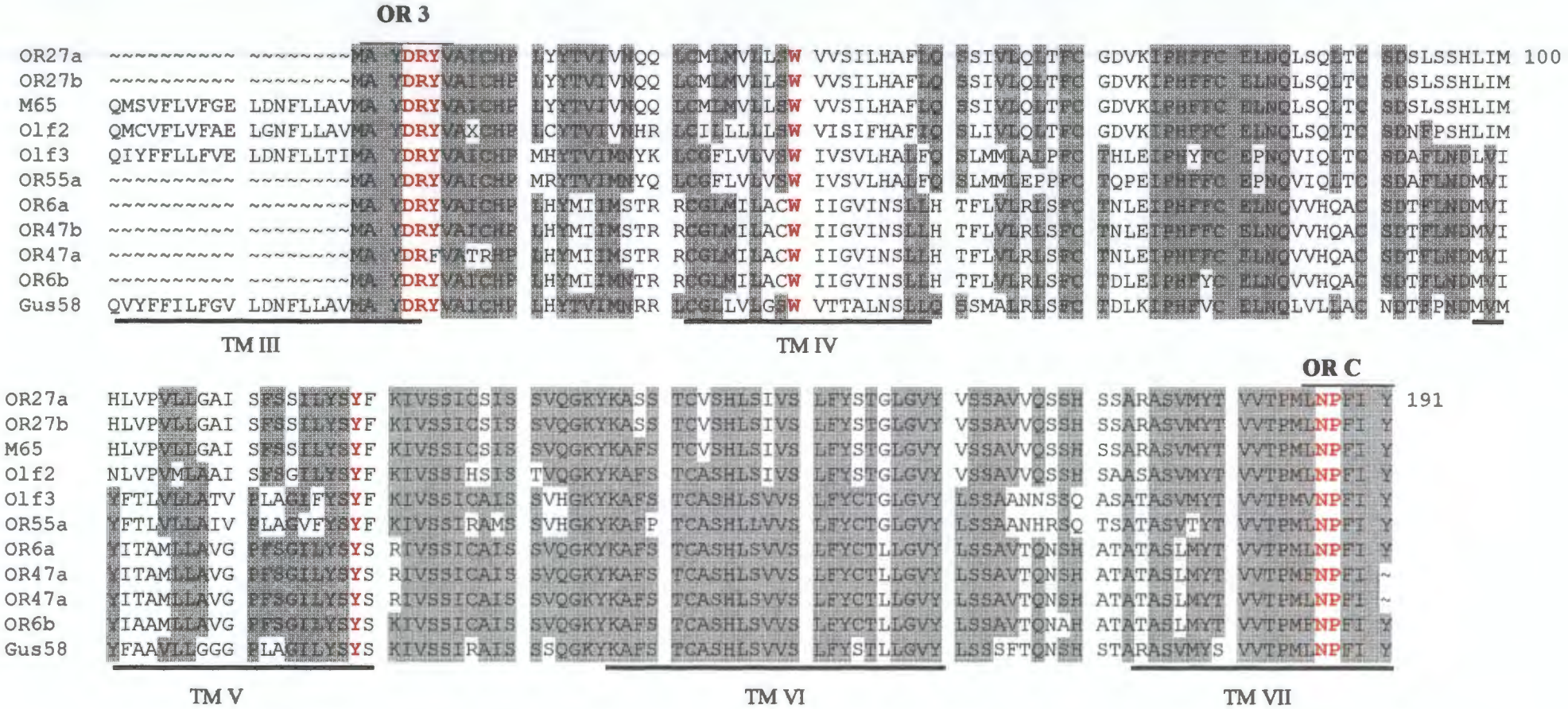


Fig 5.3 The deduced amino acid sequence encoded by the cDNA clones from the olfactory cell lines are OR27a, OR27b, OR55a, OR6a, OR47b, OR47a, OR6b. cDNA clones were subjected to DNA sequence analysis and the protein sequence encoded by each was determined. Alignment of these clones with putative olfactory receptor clones (Olf-2, Olf-3, M65) or taste receptor clones (Gus58), with which they have highest homology. Amino acid residues conserved in 60% or more of the proteins (Buck & Axel 1991) is indicated by shading. Solid black lines are predicted transmembrane domains (TM III-VI) of the olfactory G-protein family of receptors. The forward (OR 3) and reverse (OR C) primers are indicated. Conserved amino acid sequences for all the members of the olfactory receptor gene family are typed in red (Buck & Axel 1991). The greatest amino acid sequence diversity was found in the TM IV and the TM V, which form part of the ligand binding domains.

Table 5.3 *The five highest homologies to the cDNA clone sequence in the GCG database. OR6a is a representative for the clones from the OP6 and the OP47 cell line while the OR27a clone is a representative for both OR27a and OR27b clones.*

Clones	OR6a	OR27a	OR55a
M65-mouse	70%	98%	70%
Olf2-rat	61%	88%	62%
Olf3-rat	69%	66%	91%
Gus58-rat	78%	67%	67%
Gus45-rat	73%	65%	63%

Alignment of the clones from the OP cell lines with known sequences to which they had greatest homology (Fig 5.7) showed that the clones from the OP cell lines had structural similarity to other olfactory and taste receptors with 7 TM motifs. The greatest variability was found in TM IV and TM V - i.e. those regions believed to be part of the ligand binding domain (Abe *et al.* 1993b). The receptors expressed by the OP cell lines were putative 7 TM GPCRs which contained the conserved residues characteristic of this family (Buck & Axel 1991). These were the 'DRY' in the TM III, the 'W' in TM IV, the 'Y' nearest the C-terminus in TM V and the NP in TM VII which were common in all G-protein receptors (Fig 5.7).

5.4.4 *RT-PCR did not detect expression of more than one receptor type in each cell line*

Since only two of approximately 20 clones from each cell line were taken for sequencing, it was possible that, among the remaining clones there might have been transcripts of different olfactory or taste receptors in a given cell line or clones, like OR55b, that contained clusterin gene transcripts. To examine these possibilities and without going to the length of sequencing all ~ 20 clones from each cell line, I turned to restriction analysis. In this approach I used PCR with SP6 and T7 primers to amplify the inserts in the pGEM-T vector from at least 20 clones from each cell line. The ~700 bp inserts from positive clones were then digested with restriction enzymes [Fig 5.4 (A-D)] that were chosen to generate unique fragments from each of the sequenced ORF's. These enzymes are listed in Table 5.4 and their cleavage sites are indicated in [Figs 5.2 (A-D)]. The results of this analysis are summarised in Table 5.5.

Table 5.4 *Summary of restriction enzymes for clones of each cell line*

ORF from:	Enzyme	Expected cDNA band	
		bp	Fig
OR6	Sph 1	464, 231	5.6 (A)
OR27	Sac 1	488, 207	5.6 (B)
OR47	Sph 1	464, 231	5.6 (C)
OR55a	Hind III	492, 203	5.6 (D)
OR55b	Bgl II	360, 226, 97	5.7

Table 5.5 *Summary of digestion pattern for the clones of each cell line*

Cell line	pGEM-T inserts examined	Digest patterns[1]		
		Consistent	Clusterin	Unexplained
OR6	23	21	2	-
OR27	23	23	0	-
OR47	20	20	0	-
OR55	21	15	6	-

[1] Digest Patterns - A "consistent" pattern was observed when the inserts were digested with the enzyme unique for the putative receptor ORF to give the expected fragment sizes listed in Table 5.5.

This stategy provided a convenient additional check to ensure that the amplified sequences were from the cloned RT-PCR products and not from genomic DNA. Digestion of the PCR products of genomic DNA amplified with the same primers gave completely different restriction digestion patterns [Fig 5.4 (A-D)]. Except for the OP55 cell line, I have shown that the two clones isolated from each of the cell lines were virtually identical to each other, so providing some evidence for singular expression in each of the cell lines. The pGEM-T clone of OR55b contained a clusterin gene transcript.

5.5 Discussion

RT-PCR with degenerate primers designed against conserved sequences in TM3 and TM7 of the olfactory receptor superfamily, showed that the OP6, OP27, OP47 and the OP55 cell lines expressed mRNA transcripts for olfactory receptor genes. In order to exclude the possibility that the RT-PCR products were amplified from contaminating genomic DNA, I cloned and analysed them. Each olfactory receptor cell is known to express only one particular olfactory receptor. Thus, PCR amplification of mRNA transcripts should give a homogenous product. However, PCR amplification from genomic DNA would be expected to give a heterogeneous product.

RT-PCR is a very quick and useful method to identify transcripts expressed at a low level. However, one of its major shortcomings is that it is very easy to amplify false positives. Even though, the RNA used for this RT-PCR was treated with DNase, I wanted to ensure that the PCR product obtained was due to cDNA amplification and not genomic DNA contamination.

Since the coding regions of olfactory receptor gene family do not contain introns (Buck and Axel 1991, Thomas *et al.* 1996) it may be difficult to be certain that RT-PCR products represent mRNA and are not derived from genomic contamination.

I believe that genomic contamination in my experiment can be excluded since:

- The RNA was treated with DNase 1
- Only one reverse transcription reaction was performed for each sample and 1/10 of this reaction was then used for PCR with each primer pair
- If there was DNA contamination, it would be expected that multiple olfactory receptor subtypes would be amplified by PCR.
- Primers to β-actin, GnRH and Mash-1 (Table 2.4) were designed across introns which would have resulted in larger PCR products for genomic DNA contamination than for the RNA transcript - no DNA contamination was detected at this point [Fig 4.1; 4.5 (C; F)].

The results of the experiments described in this chapter may be summarised as follows:

- Four clonally derived cell lines, OP6, OP27, OP47 and OP55 could be induced to express olfactory transcripts, portions of which, when reversed-transcribed, could be amplified using, as PCR primers, sequences designed to anneal to regions of rat olfactory receptors flanking the

terminal parts of TM3 and TM7.

- Primitive, immortalised cells were induced to express the transcripts by a shift to the non-permissive temperature for the immortalising SV40 Tag and by exposure to retinoic acid at 33°C and at 39°C.
- Cell lines OP6 and OP47 were induced to express an identical transcript that had greatest homology to the rat taste receptor Gus58.
- The OP27 cells expressed a transcript which had 98% homology with an olfactory receptor gene, M65, cloned by the Mombaerts group (personnel communication) and 88% homology to Olf2 (Genbank). OP55 cells expressed an olfactory receptor that was 91% homologous to Olf3.
- At least twenty clones, derived from the RT-PCR product of each cell line, were analysed by colony PCR followed by restriction enzyme analysis. Clones derived from the OP6, OP27, OP47 and OP55 cell lines were 91%, 100%, 100% and 71% homologous respectively. The clones that differed, were shown to have the same restriction enzyme profile as clusterin.
- The sequence of the transcripts from OP55 was homologous with clusterin. Restriction analysis of several other transcript-derived clones for this cell line and two from OP6 gave results that were also consistent with clusterin having been induced in the cells (Fig 5.5).
- At the nucleotide level, clusterin is 30-40% homologous to olfactory/taste receptors (Table 5.1). but less than 9-12% at the amino acid level (Table 5.2). The forward primer, OR 3 and the reverse primer, OR C are 26% and 65% homologous, respectively, to clusterin gene. The sequenced clone OR55b was identical to mouse clusterin, except at the primer sequences. For olfactory receptor RT-PCR, degenerate primers were used at a low annealing temperature and this could have allowed amplification of clusterin, since the sequenced OR55b contained the correct forward and reverse primers for the olfactory receptor transcripts. The clusterin gene could have been amplified by mispriming of the olfactory receptor degenerate primers, since the primers are not 100% homologous to sequences in the clusterin gene.
- Restriction analysis of other RT-PCR derived clones gave results that were consistent with the belief that a particular olfactory cell expresses only one olfactory (or gustatory) receptor.
- Expression of the olfactory receptors in the cell lines adds further evidence to their validity as a model to study differentiation of the olfactory neuroepithelium.

It has been proposed that only one or a few olfactory receptors genes are expressed per cell (Buck & Axel 1991; Chess *et al.* 1994) but that the same olfactory gene could be expressed by a number of different cells. The colonies in each cell line gave predominantly the same restriction pattern providing conclusive evidence that the PCR amplification was not due to genomic DNA

contamination.

The putative olfactory receptor expressed by the OP55 cell line was only 69% homologous to olfactory receptor expressed by the OP27 cell line. This would imply that the clones from the OP27 cells and the OP55 cells represented members of different subfamilies. The olfactory receptor expressed by the OP27 cell line belonged to the same subfamily to which the Olf2-rat olfactory receptor belonged and the olfactory receptor expressed by the OP55 cell line belonged to the same subfamily as the Olf3-rat olfactory receptor. This was based on the suggestion by Ressler *et al.* (1994b) that individual genes in the olfactory receptor family shared 40-80% identity and that receptors in the same subfamily were 70-80% identical to each other.

The results merit further discussion in the following respects:

a) A taste receptor was induced in immortalised cells derived from the olfactory placode

b) Clusterin genes are inducible in cells destined to express olfactory or gustatory receptors.

5.5.1 Taste receptors

Taste transduction, triggered by a variety of gustatory stimuli, is mediated by receptors found in specialised cells in the taste buds. Taste buds are found in papillae - the blunt pegs on the surface of the tongue - and the taste receptor cells they contain have a lifespan of approximately ten days (Beidler & Smallman 1965; Farbman 1980) and are continuously replaced by basal cells in the taste bud. The taste sensory cells have no axons; information is transmitted from them through synapses onto the terminals of sensory fibres within the taste bud (Palay 1991; Sheperd 1988).

There is good reason to believe that the G-protein-mediated signalling system is involved in taste transduction, especially for sweet and bitter stimuli (Hwang *et al.* 1990; Striem *et al.* 1989). Using this information a number of researchers have been able to clone putative taste receptors (Abe *et al.* 1993 a; b; Matsuoka *et al.* 1993; Tal *et al.* 1995; Thomas *et al.* 1996) using degenerate primers to conserved regions of the G-protein-coupled receptor family.

Abe *et al.* (1993a), for example, identified six putative receptor genes belonging to the family of G-protein receptors that showed structural similarity to the olfactory gene family. In these experiments northern blot analysis showed that these taste receptors were exclusively expressed in the tongue epithelium and not in any of the other tissues tested. However, the northern blot did not include

RNA from the olfactory epithelium and it is possible that these 'taste receptors' could be olfactory receptors that were accidentally isolated from the taste buds (P. Mombaerts, personnel communications, Rockerfeller University New York). A mini-library was prepared from RT-PCR products prepared from the tongue epithelia and was screened with a probe corresponding to the conserved TM3 region of the olfactory receptor family. A number of clones were obtained and sequenced and these revealed that taste receptors belonged to a multigene family with 60 or more members. The identification of this multigene family was further confirmed by Southern blot analysis using the six putative receptor clones. Nucleotide sequencing revealed that the 60 clones were closely related but different proteins, all of which contained the 7 TM domain motifs commonly observed in the various G-protein coupled TM receptors.

When the six clones were used to screen a cDNA library prepared from rat tongue epithelium (Abe *et al.* 1993b). A full length cDNA clone, Gus27, was isolated with greatest homology (80%) to one of the above mentioned six clones - PTE33 - and to the rat olfactory receptor clone - Olf3 (56%).

However, Gus27 differed from the olfactory receptors and other G-protein receptors in several respects: The NH_2-terminal extracellular domain of Gus27 consisted of ten amino acids and was thus smaller than the corresponding domains of other GPCRs; considerable sequence differences were observed between TM IV and TM V (i.e. parts of the ligand binding regions) of the taste and the olfactory receptors; differences were also found in the intracellular domains 3 and 4, believed to be involved in intracellular signalling. Southern blot analysis confirmed that Gus27 belonged to a multigene family of taste receptors. *In situ* hybridisation and northern blot analysis indicated that the mRNA was expressed in taste epithelium and in particular in the taste buds of the fugiform and the circumvallate papillae.

In the northern blot analyses, Abe *et al.* (1993a) did not check for taste receptor expression in olfactory epithelium. However, others who have included RNA from the olfactory epithelium in their northern blot analyses, have shown that taste receptors were not exclusive to the taste epithelium but expressed at high levels in the olfactory epithelium (Matsuokia *et al.* 1993; Thomas *et al.* 1996; Tal *et al.* 1995).

Thomas *et al.* (1996) isolated three taste receptor clones from vallate taste tissue by RT-PCR using degenerate primers to the rat olfactory gene family. The three clones isolated showed greatest homology to olfactory receptor genes (30-75%). Full length clones for the PCR products were isolated by screening a genomic DNA library. In order to identify expression patterns of these

clones in different tissue, RT-PCR with specific primers were performed. These three clones were found to be expressed strongly in taste buds, olfactory epithelium and in the male reproductive tracts but not in other tissues tested. Furthermore, sequence analysis of the receptors expressed in different tissues, showed that the proteins expressed in the different tissues were identical and were derived from the same gene.

Cloning and sequencing of the PCR products derived from degenerate primers to the olfactory receptor transcripts provided strong evidence for the expression of olfactory and taste receptors in the olfactory cell lines. The function for taste receptors in the olfactory epithelium is unknown. To confirm that these clones are truly taste receptors, their ligands need to be identified.

To confirm that the taste receptors cloned from the OP6 and the OP47 cells were indeed taste receptors, full length sequences have to be determined. The NH_2 terminal domain will provide further evidence that these are taste receptors, since these are said to differ from olfactory receptors and other GPCRs in that their NH_2 terminal regions are smaller (Abe *et al.* 1993b). The clones from the OP6 and the OP47 cell lines were only 73-78% homologous to known taste receptors (Table 5.3) implying that the putative receptors cloned from the OP6 and the OP47 cell lines were novel taste receptors or that they were olfactory receptors from a different subfamily of yet unpublished olfactory receptors. A good deal of further work would be required to establish which, if either, of these possibilities is correct.

5.5.2 Clusterin

The second clone sequenced from the OP55 cell line had greatest homology with clusterin. This was amplified fortuitously as a consequence of the limited homology between clusterin and the PCR primer sequences for the olfactory receptor.

Clusterin is a glycoprotein found in many tissues and fluids. *In situ* hybridisation studies have detected clusterin expression in several tissues during embryogenesis but mainly in developing epithelia where it is associated with distinct stages of differentiation (French *et al.* 1993). Clusterin is known by many other names; sulfated glycoprotein 2 (SGP-2) (Collard & Griswold 1987), S35-S45 (Abdullah *et al.* 1988; Kierszenbam *et al.* 1988), apolipoprotein transport (apoJ) (deSilva *et al.* 1990) and testosterone repressed prostate message-2 (TRPM-2) (Smith *et al.* 1992; Zakeri *et al.* 1992).

A number of possible functions have been proposed for clusterin but the precise biological functions of this protein are uncertain. These include: initiation of neuronal degeneration (Pasinetti & Finch 1991; Pasinetti *et al.* 1993), tissue regression (French *et al.* 1994), apoptosis (Buttyan *et al.* 1989; Dragunow *et al.* 1995), cell adhesion of P19 embryonic carcinoma cells in the absence of serum (Fratelli *et al.* 1996) and a role in sperm maturation, since the highest steady state level of clusterin are detected in the caput of the epididymus (Ahuja *et al.* 1996).

Within the olfactory epithelium, clusterin is expressed as early as E12.5. *In situ* hybridisation has detected clusterin mRNA in differentiating olfactory receptor neurons and in the supporting cells in the epidermal layer closest to the mucous membrane where the protein is said to be involved in differentiation and morphogenesis of the epithelial cells and to serve as a marker for differentiation (French *et al.* 1993).

In situ studies from bulbectomized mice (Michel *et al.* 1997) revealed that clusterin mRNA is located in the lamina propria with the most intense signal in the olfactory nerve bundle, suggesting that clusterin synthesis occurs in glial cells of the olfactory nerve. These authors further hypothesised that clusterin, synthesised by glial cells, were internalised by target cells in the olfactory neuroepithelium but not produced by these target cells themselves. Clusterin may play a role in maintaining lipid homeostasis in wounded cells and may ensure solubilization of potential cytotoxic compounds in these cells.

Chapter 6

Conclusions

Conditionally immortalised cell lines from the olfactory epithelium have been generated by the introduction of a temperature sensitive SV40 Tag. The SV40 Tag is active at 33°C, but can be inactivated by a shift to the non-permissive temperature of 39°C. The main aim of this study was to obtain a neuronal cell line that could ultimately be used as an experimental tool to assay the biological effects of different growth and neurotrophic factors on the differentiation of the olfactory neuroepithelium. These factors are known to be important for neural regeneration and the treatment of neurodegenerative diseases.

The olfactory system is useful for the study of neurogenesis and the effects of different neurotrophic factors involved in this process, consisting, as it does, of olfactory sensory neurons that are true neurons with an average lifespan of 30 days. They are replaced throughout the life of an animal from globose basal progenitor cells situated in the basement membrane. The system is a relatively simple one comprising only two types of neurons (olfactory and GnRH neurons) that derive from the olfactory neuroepithelium. This is different from other regions of the brain where multiple neuronal types are found.

In this study four OP cell lines, (OP6, OP27, OP47 and OP55) have been generated by retroviral gene transfer. The cell lines were characterised morphologically and by screening for expression of different genes that are known to be characteristic of the different stages in the developmental pathway from stem cells to mature olfactory neurons.

A summary of the possible markers and the developmental pathway in the olfactory epithelium is provided in Fig 6.1. One model proposes the existence of a self-renewing stem cell [for which no molecular markers exist (Calof *et al.* 1996)] while another suggest that the horizontal basal cells (HBCs) provide the stem cells from which olfactory neurons develop (Grazaidei & Monti Grazaidei 1979, Harding *et al.* 1977). Whether the HBCs are the self renewing stem cells in the olfactory system or whether another self renewing stem cell gives rise to HBCs has not been resolved [Fig 6.1; **? 1**].

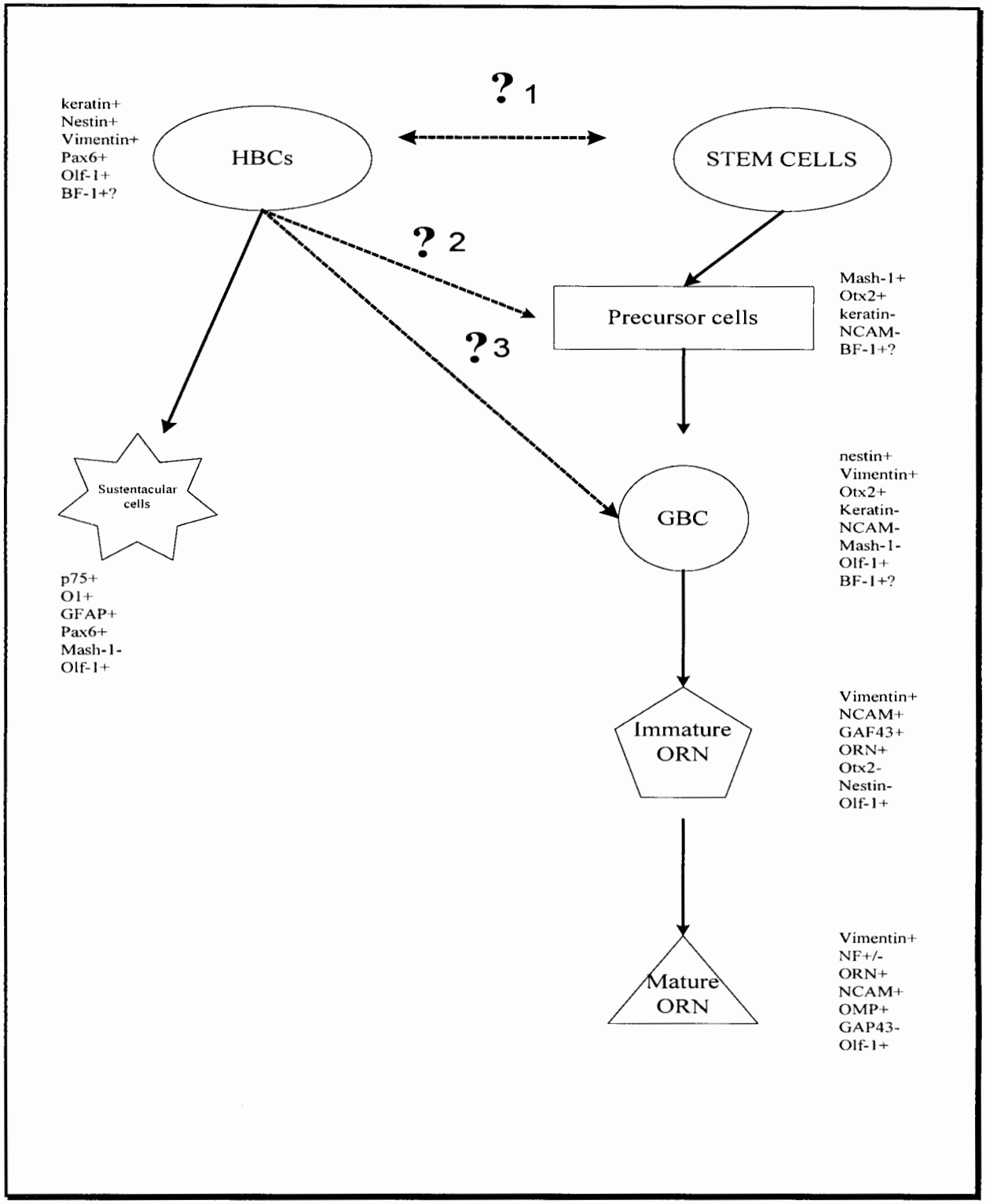


Fig 6.1 A proposed model for the pathway of the different cell types in the olfactory epithelium. Self-renewing stem cells divides to give rise to precursor cells which differentiates into GBC and further differentiates into immature ORN and then into mature ORNs. It has been suggested that the HBCs are the true precursor in the olfactory system and that they can give rise to sustentacular cells or/and cells in the olfactory neuronal lineage, either giving rise to precursor cells or to GBC. HBCs, horizontal basal cells; GBC, globose basal cells; ORN, olfactory receptor neurons. Broken lines with arrows indicate possible pathways in the olfactory pathway. The diagram is discussed in the text.

The HBCs are flat, dark basal cells which express keratin, nestin, vimentin, Pax6 and Olf-1. The lineage of the HBCs is not clear. One opinion (Davis & Reed 1996) is that the HBCs (or a subpopulation thereof) give rise to sustentacular cells, which express markers for p75, O1, GFAP, but lack expression of Mash-1 and Olf-1. Others are of the opinion that the HBCs are the true stem cells in this system. Since globose basal cells (GBC) have been shown to be the direct precursors to olfactory receptor neurons (Caggiano *et al.* 1994; Graziadei & Monti Graziadei 1978a; b; 1979; Calof & Chikaraishi 1989; Schwartz Levey *et al.* 1991; Suzuki & Takeda 1991a; 1993), HBCs should differentiate into GBC [Fig 6.1; **? 3**] or their precursor [Fig 6.1; **? 2**], which express Mash-1, Otx2, BF-1 and Olf-1 but not keratin or NCAM.

GBC express nestin, vimentin, Otx2, Olf-1 and BF-1. They do not express keratin, NCAM or Mash-1. The exact localisation of BF-1 to a specific cell type in the olfactory epithelium has not been determined but expression has been detected in the olfactory epithelium as early as E8.5 (Xuan *et al.* 1996), when the olfactory neuro-epithelium consists mainly of olfactory precursors and basal cells.

The GBC, the progeny of precursor cells, identified by Gordon *et al.* (1995), differentiate to give rise to immature olfactory receptor neurons which are postmitotic cells that loose expression of nestin and Otx2 but continues to express Olf-1, vimentin, NCAM and GAP43 (Calof & Chikaraishi 1989; Verhaagen *et al.* 1990). Immature olfactory receptor neurons undergo further differentiation to become mature olfactory receptor neurons which express Olf-1, NCAM, OMP (Margolis 1980) vimentin and olfactory receptor transcripts. They lose GAP43 (Verhaagen *et al.* 1990) expression and only a small percentage of olfactory receptor neurons has been shown to express neurofilament markers.

GnRH neurons differentiate from the embryonic olfactory placode but their cell lineage relationship to GBC or the HBCs is not known

In this thesis I have describes the immortalisation, characterisation and differentiation of four OP cell lines.

- The OP cell lines were immortalised with the temperature sensitive SV40 Tag which allows continuous proliferation at 33°C, confirmed by growth curves and expression of the SV40 protein in all cells.
- The OP cell lines were immortalised rather than transformed, since no colonies grew in soft agar.

- Each OP cell line was derived from a single clone as revealed by Southern blot analysis although morphological analysis showed the existence of more than one cell type at 39°C.
- The SV40 Tag was inactivated at the non-permissive temperature of 39°C. This was confirmed by ICC, which showed no expression of SV40 Tag protein, by cessation of growth curves and the absence of BrdU incorporation at 39°C.
- To study reversion of growth, the OP cells were grown at 39°C for 8 days and then shifted back to 33°C. Growth inhibition proved to be a reversible process. After 8 days at 39°C, a subpopulation of cells re-entered the cell cycle when shifted back to 33°C.

To induce differentiation of the OP cell lines I grew them at the non-permissive temperature of 39°C in DM-10 with or without the morphogen, retinoic acid. Expression of phenotypic markers and morphology were related to the cell lineage stages shown in Fig 6.1. Expression of olfactory receptor transcripts by all four cell lines show that they all fell into this scheme and can be summarised as follows:

OP6 at 33°C could be a GBC, some of which were induced at 39°C to differentiate into immature olfactory receptor - expressing neurons. OP27 resembled the HBCs at 33°C and a shift to 39°C induced them to differentiate into immature olfactory receptor - expressing neurons.OP47 cells could be regarded as precursor cells or GBC which differentiated to become immature olfactory receptor - expressing cells. At 33°C, OP55 cells could be keratin$^+$ HBC which differentiated once shifted to 39°C to become precursor cells, a population of which differentiated further to immature olfactory receptor - expressing immature neurons.

Shifting the OP6 cells to 39°C in DM-10, induced a change in morphology with two cell types being observed: a large, flat epithelial cell that died early in culture and a refractile, elongated cell with long extensions which grew on top of the first cell type. Under these conditions there was an increase in BF-1, Otx2 and olfactory receptor transcripts and a decrease in nestin and keratin protein expression. Nestin immunoreactivity was lost by 65% of the population of cells suggesting that these cells had started to differentiate. A subpopulation of cells continued to express the proliferating marker BF-1 and faintly expressed Otx2 - a marker which has been shown in *in vitro* studies to be a marker for basal cells and in *in vivo* studies to be a marker of mature olfactory neurons. At 33°C, 4% of the population expressed keratin but this was lost with a shift to 39°C. The transcripts for olfactory receptor genes were strongly expressed when the OP6 cells were shifted to 39°C in DM-10.

When the OP6 cells were shifted to 39°C in RA medium 50% were lost. The cells that survived lived longer than those maintained in DM-10. The shift to 39°C in the presence of RA had a different effect on the growth of the cells. The expression of BF-1 was similar to that in DM-10 but the loss of nestin expression was far less ~15%. In contrast there was a greater increase in Otx2 expression but a lower expression of the olfactory receptor transcripts suggesting that only a small population of cells differentiated from nestin+ progenitor cells to a more mature olfactory receptor-expressing cell, in the presence of RA.

In DM-10 the expression of different markers and the change in morphology with the temperature shift strongly suggest that a fraction of the OP6 cells were GBC which were induced, at 39°C, to differentiate to become immature olfactory receptor neurons. In the RA medium, the proportion of cells which followed this pathway was less - possibly only the 15% of the nestin-negative cells. In RA the GBC population was stabilised whereas in the DM-10 the majority of the cells are induced to differentiate.

An outstanding feature of the OP27 cells was their large, flat morphology prominent both at 33°C and at 39°C. At 33°C, some large cells also extended processes. This morphology together with the expression of keratin, Olf-1, BF-1, nestin and Otx2 would make OP27 cells a good candidate for a member of the HBCs population This is further supported by the increase in expression of Pax6 transcript - a marker for HBCs - at 39°C in DM-10 and in RA medium. However, a shift to 39°C in RA medium caused OP27 cells to loose Pax6 expression. Early in embryogenesis Pax6 is broadly expressed, peaking at E10.5 and then becoming restricted to HBCs and sustentacular cells. This indicates that under these growth conditions the OP27 cells differentiated from HBCs to a more mature cell type, an "immature ORN". The OP27 cells were shown to express the olfactory receptor transcript once shifted to 39°C. Sequence analysis of this transcript showed 98% homology to an mouse olfactory receptor gene (M65) which was cloned by Peter Mombaerts group (unpublished data). The continuous expression of Otx2 and the down regulation of keratin and the lack of Mash-1 expression may suggest a pathway for the OP27 cells from "HBCs" to "GBC" to "Immature ORN" (Fig 6.1).

OP47 cells grown at 39°C presented a very similar morphology to the OP6 cells but the refractile cells positioned on top of the large, flat cells, did not form a network across these cells as seen in the case of the OP6 cells. Interestingly, both OP6 and OP47 cells differentiated to express the same receptor, which had greatest homology to the taste receptor, Gus58 (78%). For the OP6 cells a shift

to 39°C alone was sufficient to induce expression of this receptor. While for the OP47 cells a shift to 39°C and the addition of RA induced expression of this receptor.

The phenotypic markers strongly suggested that the OP47 cells belonged to the "precursor cell" population or the "GBC" population. The OP47 cells lacked nestin expression at 33°C but its expression was induced once shifted to 39°C. The lack of nestin expression at 33°C would suggest that the OP47 cells were early precursor cells which had not yet started to express nestin when they were immortalised.

A shift to 39°C also resulted in the induction of Mash-1 and BF-1 transcripts (which are known to be expressed in proliferating cells) and, at the same time induced the expression of olfactory receptor transcripts and the down-regulation of Otx2 transcription.

In accord with the proposed model (Fig 6.1) a subpopulation of OP47 cells could be regarded as precursor cells or GBC which differentiated to become immature olfactory receptor-expressing cells with loss of Otx2 expression. Another population continued to express the proliferating markers, nestin, BF-1 and Mash-1. These different populations of cells would have given rise to the different morphologies present at 39°C both in DM-10 and in RA medium.

The OP55 cells differed morphologically from the other cell lines when grown at 33°C and at 39°C. A characteristic feature of the OP55 cells at 39°C in DM-10 was the presence of a large, flat cell surrounded by smaller cells which grew in groups or clumps. In RA this grouping was not seen but the two cell types were still evident.

In both DM-10 and in RA at 39°C, the same phenotypic markers were expressed to relatively the same degree. Mash-1, BF-1, Otx2 and olfactory receptor transcripts were up-regulated while nestin immunoreactivity showed a decrease in expression. The up-regulation of Mash-1, BF-1 and Otx2 and the continuous expression of nestin in ~50% of the cell population suggest that a subpopulation of cells continued to be basal cells which did not differentiate. A different population of cells then differentiated to express the more mature marker, olfactory receptor transcripts.

At 33°C, OP55 cells expressed keratin. A shift to 39°C in DM-10 resulted in the lost of keratin while a shift to 39°C in RA saw the continuous expression thereof.

The expression of different phenotypic markers suggest the presence of different cell types at 39°C. In DM-10 at 33°C, OP55 cells were HBCs (keratin+) which differentiated once shifted to 39°C to become precursor cells (keratin$^-$/ Mash-1$^+$/ BF-1$^+$/ Otx2$^+$) cells, a population of which differentiated further to become olfactory receptor-expressing "immature ORN" lacking the early stem cell marker, nestin.

In RA at 33°C, OP55 cells were keratin$^+$ HBCs and a shift to 39°C maintained a population of cells which continued to express keratin. Another population of cells differentiated to a more mature phenotype.

From the results of the OP55 and the OP6 cell lines we see that the RA appeared to stabilise a basal cell population to a greater degree than the DM-10 and that more differentiation was induced in DM-10 whereas in OP47 cells, RA induced differentiation more than in DM-10. For OP27 there was no significant difference between the different growth conditions.

As discussed in Chapter 4, *in vivo* and *in vitro* studies have shown how RA plays an important role early in embryogenesis and influences the morphogenesis of the mammalian olfactory pathway. In these *in vitro* studies, the effect of RA was not considerably more effective than a shift to 39°C under standard growth conditions.

The four immortalised, clonal cell lines from the olfactory placode that I report in this thesis comprise cells that can be induced to differentiate into olfactory receptor expressing neurons. They thus serve as a useful future resource for studying olfactory receptor gene expression. The remaining 54 OP cell lines could be used for the isolation and cloning of novel olfactory receptor genes, to isolate taste receptors expressed in the olfactory epithelium and, possibly, to determine their functions in olfaction.

The cell lines provide a useful *in vitro* system to search for ligands for these receptors and to establish their nature as true receptors which respond to ligands by causing an electrical response (pulse clamp analysis) or activating a second messenger system, e.g. cAMP or inositol-1,4,5-triphosphate.

These cell lines consist of a clonal cell populations which could be grown in large quantities useful for the generation of cDNA libraries. These could be used to screen for different receptors (olfactory or taste) expressed in the olfactory epithelium. The establishment of an olfactory neuronal

cell line should permit an analysis of the mechanisms involved in selective gene expression in the odorant receptor multigene family.

These cells might be used as *in vitro* models for identifying growth factors, neurotrophic factors and different substrates which may be involved in the differentiation, maturation and survival of olfactory receptor neurons from basal cells.

Since the OP cell lines express different markers thought to be important for brain development it is distinctly possible that, during differentiation, they would express other novel gene products of relevance to ontogenesis and neural development. A consistent and well characterised panel of inducible cells would be invaluable for identifying such genes.

These cell lines could serve as a useful model to study gene therapy since the SV40 Tag is inactivated at 39°C, the body temperature of rodents and the cell lines can be transplanted in the CNS.

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