Cochlear hair cell fate determination and differentiation *in vitro*

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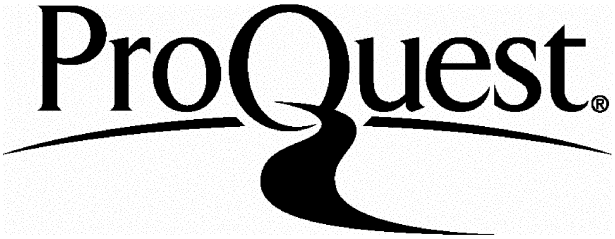

SUMMARY

Mammalian cochlear hair cells are relatively inaccessible and few in number. This hampers any research on their fate determination and differentiation. The production of conditionally immortal cell lines from the H2K^{b}*ts*A58 transgenic mouse should overcome these difficulties.

The aims of the present study were threefold. Firstly, to establish that the cell lines provide a viable *in vitro* system, by examining the pattern of molecular expression in the cochlear hair cell line UB/OC-1. Secondly, to examine differentiation by using clonal derivatives from the heterogeneous cell line UB/OC-1. Thirdly, to explore the process of lateral specification in the determination of cell fate and to explain the differentiation of hair cells and supporting cells from a common precursor.

The methods used were cell culture, immunocytochemistry, reverse transcription polymerase chain reaction and western blot. The results demonstrated that firstly; the temporal expression pattern of Brn3.1, an essential transcription factor required for hair cell differentiation, and the α9 subunit of the nicotinic acetylcholine receptor, followed a similar pattern to that during normal development. Secondly, epithelial cell markers such as, vimentin, cytokeratin, actin and cadherin, and specific hair cell markers such as myosinVIIA and fimbrin were expressed when the hair cells differentiated. The pattern of expression suggested parallel pathways of gene expression during differentiation of hair cells. Thirdly, from the expression of Numblike, Notch1, Jagged1 and *jagged2,* factors which are known to be involved in lateral specification, a model is proposed to explain hair cell fate determination.

The results also demonstrate the much greater experimental flexibility offered by cell lines in understanding hair cell development. Future studies will focus on functional experiments that alter hair cell fate.

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I performed all the experimental procedures used in this study, of cell culture, mRNA and protein extraction, cDNA synthesis, RT-PCR, western blot and black and white film development.

CONTENTS

FIGURES

Chapter One

Chapter Two

Chapter Three

CHAPTER ONE

INTRODUCTION

1.1 Deafness; the Problem

Deafness remains one of the great challenges of the 21st century. The morbidity associated with sensorineural hearing loss ranges from minor annoyance to the impairment of learning, social interaction and safety. Causative factors responsible for this affliction are varied, and include genetic defects, drugs, toxins, infectious agents and the normal ageing process. With approximately 10% of the global population affected (Rubel, 1997) and the knowledge that every second person over the age of 75 will to some degree be deaf (U.S. Department of Health and Human Services, 1994), the need to understand the ontogeny of the auditory system is paramount. For this will provide a basis to understanding the necessary biological mechanisms that will one day ensure that deafness is defeated.

In order to place this work in context in understanding the molecular biology of development within the cochlea it is necessary to give a brief overview of the structure of the mammalian cochlea and the specialised characteristics of sensory hair cells. This is followed by a review of cochlear development and in particular hair cell fate determination and differentiation. The issue of hair cell regeneration is briefly discussed, as are current methodologies for *in vitro* preparations. Finally, the use of cell lines is explored and the aims of this research are described.

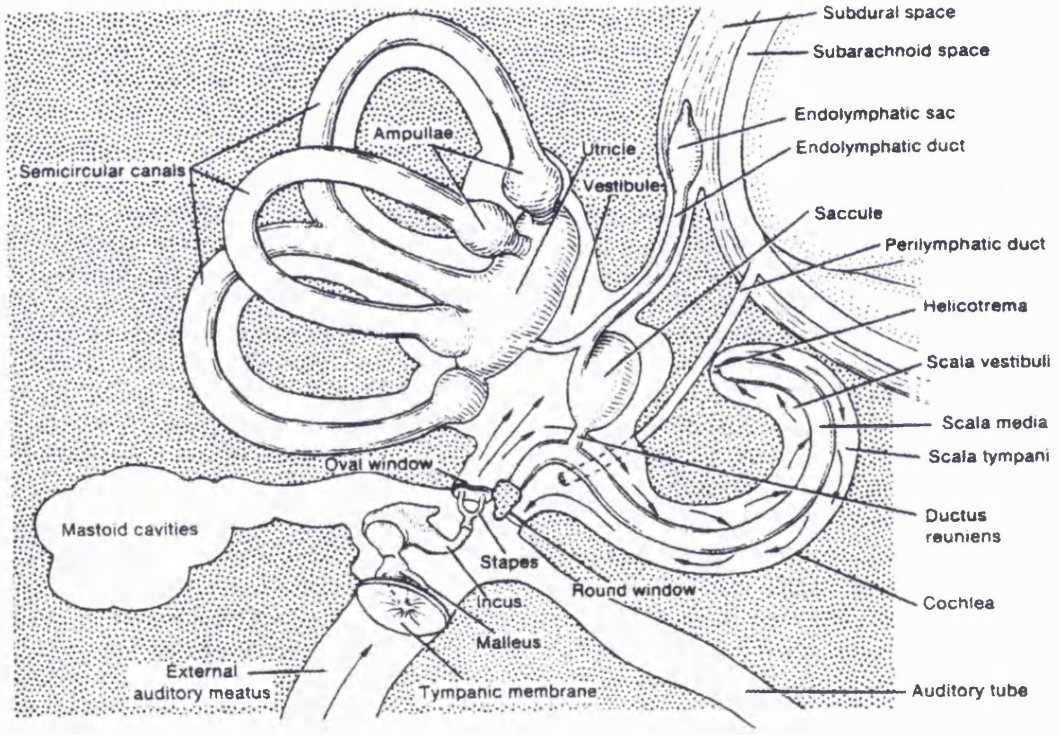
1.2 Structure of the mammalian cochlea.

The vertebrate inner ear is a complex sensory organ responsible for the senses of balance and hearing (Fig.1.1). It is composed of a bony labyrinth, the otic capsule that encases the membranous labyrinth, which contains the cellular structures. These structures include a closed epithelial layer that is diversified into specific regions containing the sensory-transducing cells, the sensory hair cells, as well as supporting and secretory cell types. Hair cells are specialised mechano-receptors organised in a sophisticated topographical pattern. Within the cochlea they are surrounded by perilymph and covered at their apex by endolymph. The potassium-rich endolymph, produced by the stria vascularis, is essential for the process of transduction by the hair cells. The endolymph contains ions, which provide the necessary voltage and ionic gradient that can change the resting potential of the hair cell. This in turn results in transmitter release from the hair cell and excitation of the afferent nerve fibres. The perilymph, within the scala vestibuli and scala tympani, is separated from the endolymph, within the scala media, by tight junctions between adjacent epithelial cells. The organ of Corti, which contains the specialised epithelial cells; the hair cells and supporting cells, spirals along the basilar membrane (BM), between the apex and the base of the cochlea (Fig.1.2).

In the human/mouse there are approximately 16000/3000 cochlea hair cells respectively (Echteler *et al.* 1994) in each ear, which are separated from one another by various types of supporting cells. The organ of Corti contains one row of inner hair cells (IHCs) medially and three rows of outer hair cells (OHCs) laterally. Hair cells and their stereocilia (Echteler *et al.* 1994) increase in length between the base and the apex of the cochlea, whilst the BM becomes thinner and wider.

FIGURE.1.1

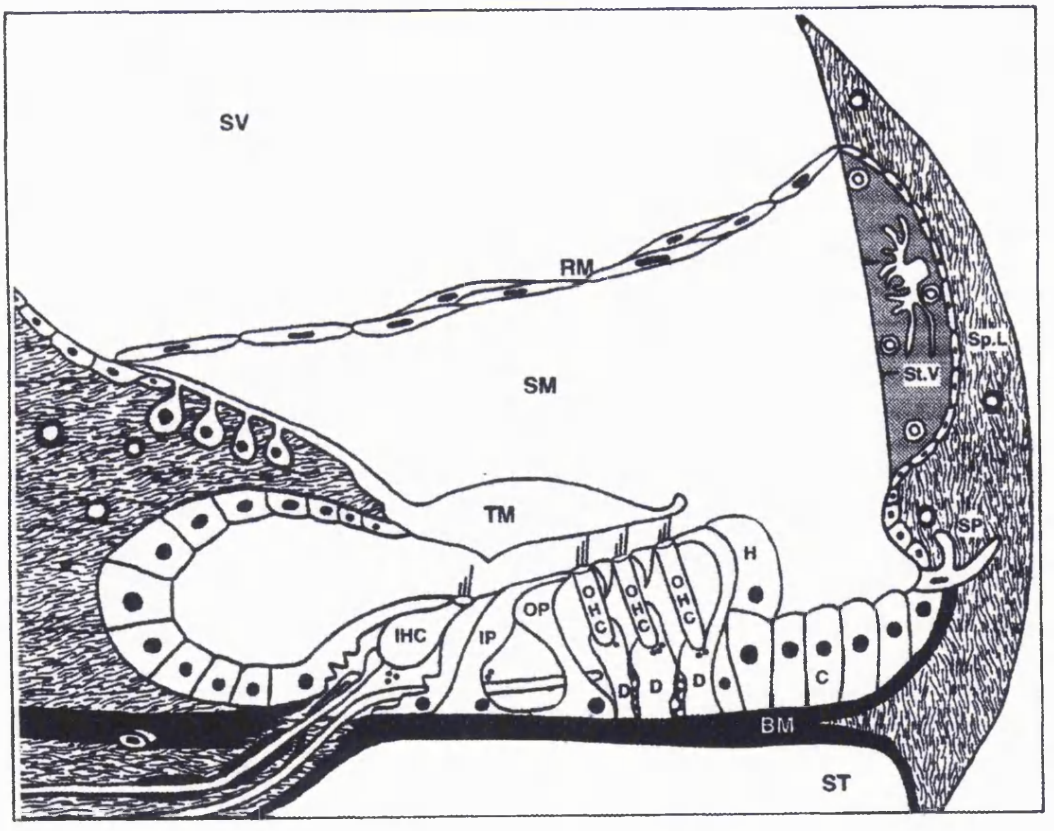
Gross anatomy of the mammalian inner ear. Depicted in detail is the membranous labyrinth, a connected system of ducts and pouches that contains the vestibular and auditory end organs. Surrounding the membranous labyrinth is the bony labyrinth. The arrows indicate the direction of flow of perilymph following inward direction of the oval window.



(Source: Echteler, et al. 1994. Structure of the mammalian cochlea. In Comparative Hearing: Mammals. Fay, R., Popper, A (eds). Vol.4.134-171. Springer, New York).

FIGURE.1.2

The adult organ of Corti. The inner hair cells (IHC) and three rows of outer hair cells (OHC), along with the inner pillar (IP), outer pillar (OP), Deiters (D), Hensen (H), and Claudius (C) cells rest on the basilar membrane (BM). The tectorial membrane (TM) covers the apical surface of the sensory and supporting cells. The scala vestibuli (SV), and scala tympani (ST) are filled with perilymph. The scala media contains endolymph and its boundaries include the reticular lamina of the organ of Corti, Reissner's membrane (RM), and the lateral wall made up of the stria vascularis (St.V), the spiral ligament (SpL), and the spiral prominence (SP).

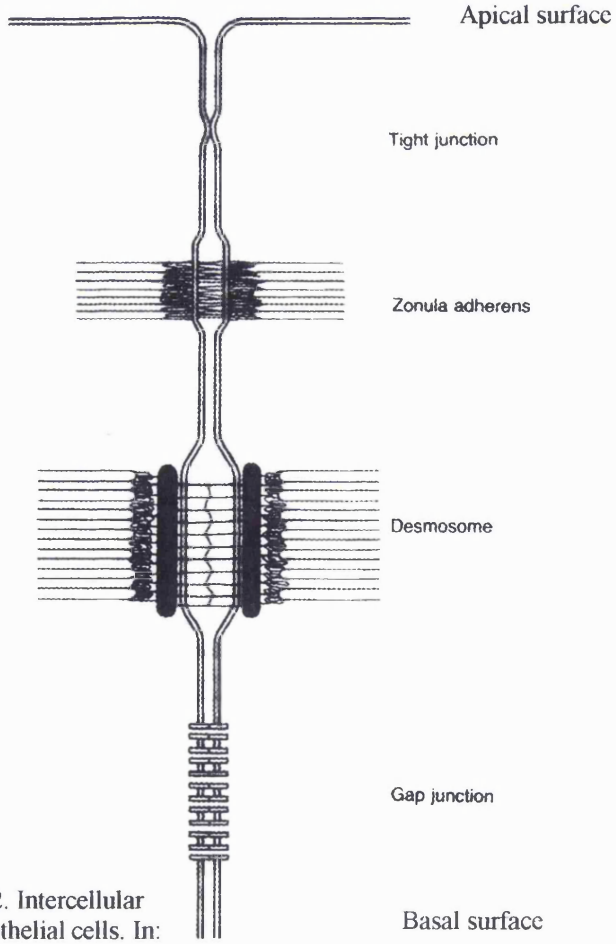


(Source: Slepecky, N. 1996. Structure of the mammalian cochlea. In: The Cochlea. Dallos, P., Popper, A., Fay, R (eds). Vol. 8. 46-129. Springer, New York.)

These morphological characteristics, in particular the stiffness of the BM, relate to the tuning capacities and sensitivities that enable such a diverse frequency range to be detected from high frequencies in the base to low frequencies in the apex. Tight junctions between hair cells and supporting cells enable them to separate the endolymph from the perilymph. Other junctions include zonula adherens junctions, desmosomes and gap junctions (Slepecky N. 1996). Tight junctions and zonula adherens junctions are present at the apical surface of the cells, whilst desmosomes and gap junctions are found along the basolateral surfaces (Fig.1.3).

FIGURE.1.3

Diagram showing the four principal types of intercellular junction that occur between two simple epithelial cells.



Source: Garrod and Collins. 1992. Intercellular junctions and cell adhesion in epithelial cells. In: Epithelial organisation and Development. Fleming (ed). 1-35. Chapman&Hall, London.

Intercellular junctions play an integral part in determining the polarity of epithelial cells. In turn, epithelial polarity influences not only membrane topography and composition, but also the internal organisation of the cell, including that of the membrane skeleton and the cytoskeleton.

Tight junctions (zonula occludens) have two major functions: firstly, they create a barrier to the diffusion of membrane molecules in the plane of the lipid bilayer, and secondly, they seal the intercellular space preventing the passage of electrolytes and macromolecules between the apical and basolateral surfaces of the epithelium. In the organ of Corti they create a barrier between endolymph and perilymph. They are found between the hair cells and supporting cells and between supporting cells. A number of proteins associated with the cytoplasmic aspect of tight junctions have been identified in the organ of Corti, namely, Zonula Occludens 1 (ZO-1) (Stevenson *et al.*, 1986), Cingulin (Citi *et al.*, 1988, Raphael and Altschuler 1991).

Adherens junctions are found in a sub-apical ring beneath the tight junctions physically binding the cells together and mediating intercellular communication. They have three domains: cytoplasmic, intramembrane, and extracellular. At the cytoplasmic domain, the junction is linked to the intracellular actin cytoskeleton, which forms a circumferential ring within the cell, by vinculin (Geiger *et al.*, 1985) and α-actinin (Lazarides and Burridge, 1975). The intramembrane and extracellular domains are composed of adhesive molecules from the cadherin family of calcium-dependent glycoproteins (Takeichi, 1988, 1990). This family includes E-cadherin (uvomorulin) (Yoshida and Takeichi, 1982), N-cadherin (Hatta *et al.*, 1987), P-cadherin (Nose *et al.*, 1987), and M-cadherin (Donalies *et al.*, 1991). E-cadherin is expressed in mature supporting cell/supporting cell adherens junctions (Whitlon, 1993), whilst ACAM (the chick homologue of N-cadherin) is expressed between hair

cells and supporting cells and in supporting cell/supporting cell junctions (Raphael *et al.*, 1988).

In the organ of Corti, desmosomes (macula adherens) are not found between hair cells and supporting cells (Gulley and Reese, 1976). Likewise, gap junctions, which are composed of connexins, are not found between mature hair cells and supporting cells. Although present in the otocyst, they are subsequently lost prior to development of hair cells in the chick (Forge, 1997). However, gap junction-like structures have been noted at the lower boundaries of the tight junctions between hair cells and supporting cells (Nadol, 1978). Recently, the gene encoding the gap-junction protein connexin 26 has been liked to the autosomal recessive non-syndromic sensorineural deafness (DFNB1) (Kelsell *et al.*, 1997).

1.4 Hair Cell Structure

Intermediate filaments (IF), microfilaments and microtubules form an intracellular structural framework called the cytoskeleton. IF have been studied extensively and provide useful markers for cell differentiation as their expression pattern tends to be tissue specific. The diameter of an IF is 8-12nm and falls between that of the smaller actin filaments and larger microtubules (Steinert *et al.*, 1984). IF have been divided into six classes based on differences in their solubility, antigenicity and electrophoretic mobility (Lazarides, 1982; Wang *et al.*, 1985). The currently recognised classes of IF are desmin, vimentin, cytokeratins, neurofilaments, nestin and glial filaments. Within the inner ear, in particular the hair cells, the expression of IF's cytokeratin and vimentin is variable. Kuijpers *et al.* (1992) demonstrated that in the rat inner ear the expression of vimentin decreased in hair cells from embryonic day 15 (E15) to maturation, but persisted within the supporting cells, external sulcus

cells and mesenchyme with trace amounts present in the adult hair cells. Similarly, the limited expression of cytokeratin in the developing sensory epithelia decreases after E12.5 (Kuijpers *et al.*, 1991b). The transient co-expression of vimentin and cytokeratin within the developing epithelia may play a role in differentiation and can be used as an early marker of differentiation (Viebahn, *et al.*, 1997). Glial filaments and desmin have not been observed at any point in the development of hair cells in the guinea pig, rat or human (Raphael, 1987; Kuijpers *et al.*, 1991b: Oesterle *et al.*, 1990). More than most other cells, hair cells rely on actin. Actin is present in at least four regions of the hair cell (Flock and Cheung, 1977; Slepecky and Chamberlin, 1982; Drenckhahn *et al.*, 1991). The majority of actin is found in the stereocilia, where it is present as bundles of parallel filaments that extend into the cuticular plate. Apically, it forms a circumferential ring of filaments associated with the adherens junctions at the reticular lamina. It is also a component of the cortical lattice just inside the cell membrane along the lateral wall and it is found in the cytoplasm (Holley *et al.*, 1992). Each of these regions has different actin–binding proteins which include profilin (Flock et al., 1982), tropomyosin (Slepecky and Chamberlin, 1985b), spectrin (Holley and Ashmore, 1990; Ylikoski *et al.*, 1990), α-actinin (Slepecky and Savage, 1994), and fimbrin (Matsudaira *et al.*, 1983). Co-localisation studies show that fimbrin cross-links the highly organised parallel actin filaments in the stereocilia of both IHC's and OHC's (Drenckhahn *et al.*, 1991). This cross-linking contributes to the stiffness of the stereocilia. In the rat, fimbrin is first noted at E18 in IHCs and by birth it is present in OHCs (Zine *et al.*, 1995). It is an essential part of the formation and ontogenesis of stereocilia.

The isoenzyme myosin VIIA, an unconventional myosin, is another important protein necessary for the development of stereocilia (Hasson and Mooseker, 1995). It

maintains the structural integrity of the hair bundles by cross-linking between adjacent stereocilia rootlets within the cuticular plate and is also found in the pericuticular necklace and cell body (Hasson *et al.*, 1997). If myosin VIIA is non-functional as in the *shaker-1* mutant mouse (Gibson *et al.*, 1995) then the stereocilia are disrupted and deafness and vestibular dysfunction ensues. In the human, mutations of the myosinVIIA gene cause Usher syndrome 1B (Weil *et al.*, 1995) and the non-syndromic deafnesses DFNB2 and DFNA11 (Weil *et al.*, 1997; Lui *et al.*, 1997c). A developmental study of myosinVIIA showed that its' mRNA is first expressed at E9 in the otic vesicle (Sahly *et al.*, 1997). At this stage, the presumptive sensory hair cells and supporting cells, both of which develop microvilli, cannot be distinguished. As cell differentiation proceeds, myosin VIIA expression is restricted to the cochlea and vestibular hair cells. It can, along with fimbrin, be used as a specific hair cell marker as it is not found in supporting cells.

1.5 Supporting Cell Structure

In supporting cells, organised bundles of microtubules and actin filaments pass from the reticular lamina to the BM. These are thought to play a role in mechanical support and coupling of the BM to the reticular lamina. Both cytokeratin and vimentin have been shown to be present in adult supporting cells (Raphael *et al.*, 1987; Bauwens *et al.*, 1991; Kuijpers *et al.*, 1991b). This is unusual as it is rare to find these two IF in the same cell and vimentin is normally only found in cells of mesenchymal origin. Specific proteins that are found only in supporting cells include the calcium binding protein S-100 (Foster *et al.*, 1994; Pack and Slepecky, 1995), and organ of Corti protein 2 (OCP-2) (Yoho *et al.*, 1997). OCP-2 is a cytosolic protein that is thought to

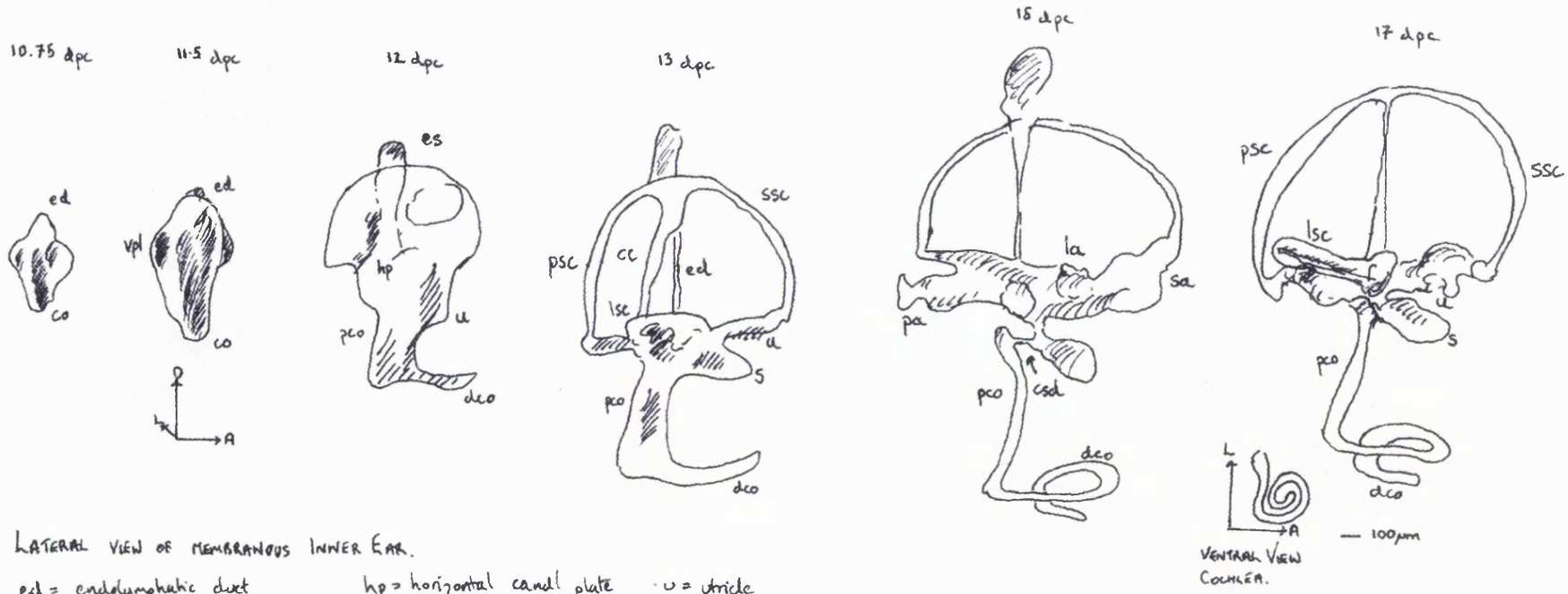
regulate in some manner the intracellular or transcellular K^+ flux in the supporting cells.

1.6 Molecular development of the murine cochlea

The inner ear develops from a thickened patch of ectoderm called the otic placode, which invaginates by E9 to form the otic vesicle or otocyst (Noden and Van De Water, 1986) (Fig.1.4). The epithelium of the otic placode also gives rise to the primary neurons of the statoacoustic ganglion or what is later termed the otic/cochleovestibular ganglion. This can be watched by time lapse videomicroscopy in the transparent zebrafish embryo where the neuroblasts crawl out individually from the ventral region of the otocyst epithelium into the connective tissue below (Haddon and Lewis, 1996). It is thought that placodes originally evolved from primordial primary sensory cells into primordia. Primordia contain both sensory cells and ganglia that connect these axonless sensory cells to the brain (Fritzsch, 1993). Development of the otocyst depends on gene expression in the rhombencephalon (the hindbrain), the nonneural portion of the otocyst, the neuronal precursors derived from the placode and the neuronal, glial, schwann sheath and melanocyte precursors derived from the neural crest (Fritzsch *et al.*, 1997). Many of these genes are required for axis specification, segmentation, and body plan patterning (Keynes and Krumlauf, 1994). Such a bewildering array of genes include the important homeobox genes which are thought to play a determinant role in the development of the hindbrain, branchial arches, vertebrae, and limbs (Keynes and Krumlauf, 1994). They are so named as they encode transcription factors which contain a highly conserved sixty amino acid DNA-binding domain; the homeodomain (Keynes and Krumlauf, 1994).

FIGURE.1.4

Schematic representation of the developing inner ear of the mouse from E.10.75 to E.17. (Source; Morsli *et al.*, 1998).



LATERAL VIEW OF MEMBRANOUS INNER EAR.

ed = endolymphatic duct
co = cochlea
vpl = vertical canal plate
hp = horizontal canal plate
pco = proximal cochlea
dco = distal cochlea
es = endolymphatic sac
u = utricle
s = saccule
ssc = superior semicircular canal
psc = posterior semicircular canal
lsc = lateral semicircular canal
pa = posterior ampulla
la = lateral ampulla
sa = superior ampulla
csd = cochleosaccular duct

Homeobox *(Hox)* genes that are involved in the development of the otocyst include; *Hoxa1* (Lufkin *et al.*, 1991; Chisaka *et al.*, 1992); *distal-less* (*dlx-3*) (Ekker *et al.*, 1992); those related to *Drosophila muscle segment* homeobox genes (*Msx-1, Msx-2*) (Suzuki *et al.*, 1991; Davidson, 1995); *NK*- related homeobox genes (*Nkx5.1, Nkx5.2*) (Rinkwitz-Brandt *et al.*, 1995); *Drosophila* orthdenticle related genes (*Otx1* and *Otx2* (Simeone *et al.*, 1993) and paired-box transcription factors (*Pax2* and *Pax3*) (Nornes *et al.*, 1990; Hoth *et al.*, 1993; reviewed in Rivolta, 1997).

Secreted factors such as *wingless-int* (*Wnt*) (Parr *et al.*, 1993), *fibroblast growth factors* (*fgf3*) (Wilkinson *et al.*, 1989), and *bone morphogenetic protein* (*Bmp*) (Oh *et al.*, 1996) are also expressed at the otocyst stage. Similarly, the zinc finger genes; *Msal,* the mouse homologue of the *spalt* gene from *Drosophila* (Ott *et al.*, 1996), *GATA-3* (George *et al.*, 1994) and the thyroid hormone receptors (*TRα1, TRα2,TRβ1* and *TRβ2*) (Bradley *et al.*, 1994) are all expressed in the otocyst. The combinatory action of all these genes is to determine the axis orientation (*dlx-3, fgf3*), proliferation (*fgf3*), formation of the inner ear (*Hoxa1, Pax2, Msx-1, Msx-2, Otx1*, and *Nkx5.1*) and cranial nerves VII, VIII (*Hoxa1*), and migration of neural crest precursors (*Pax3*).

Axis orientation in the anteroposterior, dorsoventral and medial lateral planes relies on the close spatial proximity of the hindbrain and the subsequent gene expression as described above. The cells within the otocyst then undergo proliferation and segregation of the sensory epithelia. The cochlea and saccule are derived from the ventral half of the otocyst at E11, whilst the semicircular canals and utricle are derived from the dorsal half (Li *et al.*, 1978) (Fig.1.4). Only after E11.5 is the otocyst completely autonomous in its differentiation (Van De Water, 1983). It appears that from studies conducted by Li and McPhee (1979) and Van De Water (1983) that the periotic mesenchyme is essential for cochlear formation until E11. Growth factors,

such as insulin and insulin-like growth factors (IGFs) derived from the mesenchyme cause mitogenesis in the epithelium (Leon *et al.*, 1995b). In particular IGF-1 appears to act on restricted areas of the epithelium, which may account for the observed regional differences in growth rate. Associated with the mitogenic activity of IGF-1 is the rapid induction of *c-fos* (Leon *et al.*, 1995a). *c-fos* is an essential element in the propagation of mitogenic signals regulating the expression of secondary genes, which eventually lead to cell division (Morgan and Curran, 1991). In contrast, low concentrations of retinoic acid (RA) have been shown to inhibit cell proliferation and stimulate sensory and secretory cell differentiation (Represa *et al.*, 1990). Kelley *et al.* (1993) provided further supporting evidence for this role. They demonstrated that exogenous RA added to the otocyst could produce supernumary hair cells. Importantly, if the RA was added at the time of terminal mitosis then more hair cells and supporting cells were generated.

1.7 Hair cell fate determination

Relatively little is known about the exact nature of events that leads to a particular cell fate in the inner ear. However, recent research is beginning to unravel this complex story. The initial steps of axis orientation and compartmentalisation in the otocyst appear to be crucial for subsequent cell fate specification (Fekete, 1996). Studies in mutant zebrafish, such as *dog-eared, colourless* and *van gogh* (Whitfield *et al.*, 1996) have shown that hair cells are able to form in grossly malformed ears that lack any obvious morphological compartments. This suggests that sensory patch specification does occur before gross morphogenesis. In the otocyst, the differential expression of genes at the compartment boundaries probably accounts for the distribution of the sensory patches. Therefore, cells in the dorsolateral quadrant of the otocyst will

become the cristae, whereas those in the ventral half will become the maculae and cochlear duct.

As yet in the mammalian inner ear, there is no data on how the fate of the sensory cell progenitors is determined. However, research on neurogenesis (Henrique *et al.*, 1997; Lewis, 1996 for review), myogenesis (Kopan *et al.*, 1994) and haematopoesis (Li *et al.*, 1998) has led to an understanding of some of the mechanisms involved. In neurogenesis of *Drosophila*, the first step to take place is the switching on of proneural genes in a cluster of cells. These genes form the *achaete-scute* complex (Ghysen and Dambly-Chaudiere, 1988) and endow the cells that express them with the potential to form neural precursors. The *achaete-scute* complex, termed *Mash* (*mammalian achaete-scute homolog*) in mammals (Johnson *et al.*, 1990), encode transcriptional regulators of the basic helix-loop-helix (bHLH) type. Another important bHLH gene is *atonal* (*Math, mammalian atonal homolog*, in mammals (Akazawa et al., 1995)). In *Drosophila, achaete-scute* and *atonal* do not only act as proneural genes for their respective lineages but they also help to determine the type of sensory organ developed ie; either an external sensory organ (sensory bristle) or photoreceptor (Jarman *et al.*, 1993b). Once the proneural cluster has been determined and the particular bHLH genes switched on then the process by which one cell within the cluster is singled out to follow a particular fate depends on both extrinsic and intrinsic mechanisms.

The intrinsic mechanism, at least in part, relies on the gene *numb* (Uemura *et al.*, 1989; Rhyu *et al.*, 1994). Numb protein is membrane associated and asymmetrically localised in the shape of a crescent in the sensory organ precursor of *Drosophila* prior to cell division. Upon division, Numb protein is preferentially segregated into one of the daughter cells indirectly determining its fate (Rhyu *et al.*, 1994). Studies of *numb*$^{-}$

mutants have shown that cell fate can be altered by changing the level of expression of *numb*. Furthermore, *numb* is able to generate asymmetry in the subsequent division of the daughter cell. This appears to be a general feature of asymmetric cell divisions in the fly nervous system. In the mouse, Zhong *et al.*, (1996) have found a *numb* gene that shares a high degree of sequence homology with fly *numb*. Mouse Numb protein is also membrane associated prior to cell division, but unlike fly Numb it can be distributed either symmetrically or asymmetrically in the daughter cells. This difference may reflect the different strategies the fly and mouse have adopted during evolution to fulfil neurogenesis. In the fly, except in the eye, all the neural precursors undergo asymmetric cell divisions to generate neurons. In the mouse, neural precursors undergo symmetric cell division to increase the size of the precursor pool before undergoing asymmetric cell division to generate neurons (Anderson and Jan, 1997). Mouse *numblike*, which also has significant sequence similarity to *Drosophila numb* has been found to be symmetrically distributed in dividing neural precursors (Zhong et al., 1997). Nevertheless, its action is similar to that of *numb* which is to antagonise Notch signalling activity.

The extrinsic mechanism relies on the interactions between members of the Notch and Delta/Serrate/Lag family of proteins (Hartenstein and Posakony, 1990; Goriely *et al.*, 1991; Heitzler *et al.*, 1991). Notch1-4 are cell surface receptors that are activated through direct cell surface contact with a ligand expressing cell. Delta1,2,3/Serrate1,2/Jagged1,2 are the ligands (Dunwoodie *et al.*, 1997; Lindsell *et al.*, 1995). Notch/ligand (eg.Jagged) are the mediators of "lateral inhibition/specification" (Greenwald, 1998). This is a critical signalling system that enables one cell to follow a particular fate, whilst its neighbours follow a different fate. Activation of the Notch receptor by the ligand leads to suppression of

differentiation within that cell. It appears to be a universal system in possibly all multicellular organisms in a variety of developmental processes (Chitnis *et al.*, 1995). Once Notch has been activated its intracellular domain is cleaved by a protease (Schroeter *et al.*, 1998). This domain then interacts with a transcription factor named Suppressor of Hairless (Su[H]) in *Drosophila* and RBP-Jk/KBF2/CBF1 in mammals; collectively known as the CSL proteins (Tamura et al., 1995; Jarriault et al., 1995). Prior to induction by Notch, the CSL proteins interact with a corepressor complex containing SMRT (Silencing Mediator of Retinoid and Thyroid hormone receptors) and the histone deacetylase HDAC-1. Notch disrupts the formation of the repressor complex such that the CSL protein now becomes an activator of transcription (Kao *et al.*, 1998). The Notch/CSL protein complex drives the transcription of a group of related genes known as *Enhancer of split (E[spl])* in *Drosophila* and *Hes* (Homolog enhancer of split) in mammals (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). These genes encode bHLH transcription factors which combine with a protein called Groucho to create a repressor (Paroush *et al.*, 1994). Finally, the *Hes*bHLH/Groucho protein complex represses genes of the *achaete-scute* complex (Oellers *et al.*, 1994; Heitzler *et al.*, 1996) thereby blocking differentiation. As the Ac-Sc proteins activate the expression of *Delta* (Kunisch *et al.*, 1994), this feedback loop operates to repress *Delta* expression in the cell in which Notch has been activated. Thus, lateral specification appears to be regulated through the Notch → RBP-J →HES → Math pathway. Furthermore, Notch signalling through the expression of HES can also upregulate the expression of Notch itself (Weinmaster, 1998). This positive regulation of Notch would serve to reinforce the cell's responsiveness to Notch ligands expressed by surrounding cells.

Taken together with the activity of *numb*, Jan and Jan (1995) have proposed that Notch mediated signalling is influenced by *numb* at each successive step in the sensory organ precursor (SOP) lineage. Essentially by antagonising Notch, *numb* creates a bias between sister cells that signal each other. They suggest this as a mechanism to increase the reliability of Notch-mediated signalling in situations requiring rapid decision making, when there may be insufficient time to activate transcription-based feedback mechanisms. The mechanism by which Numb antagonises Notch is not yet known, although one possibility is that Numb interferes with the interaction of Notch with RBP-J (Frise *et al.*, 1996).

Other proteins also modulate the activity of Notch and its ligands. This has been found during wing formation in *Drosophila* where activation of the ligands, Serrate and Delta, is modulated by Fringe (Irvine and Wieschaus, 1994). Fringe can inhibit Serrate signalling through Notch yet enhance Delta activated Notch in a cell autonomous fashion (Panin *et al.*, 1997). This differential signalling through Notch is crucial in establishing the dorsal-ventral boundary of the wing margin, which is required for morphogenesis and patterning of the wing. The mammalian homologues of *fringe; lunatic, manic* and *radical fringe* have recently been isolated (Johnston *et al.*, 1997; Cohen *et al.*, 1997). *Lunatic fringe* is required for boundary formation (Zhang and Gridley, 1998; Evrard *et al* 1998) and symmetric patterning. In the murine inner ear, *lunatic fringe* was first expressed at E9.5 in the anteroventral aspect of the otocyst (Morsli *et al.*, 1998). By postnatal day 1 (P1) *lunatic fringe* transcripts were restricted to the supporting cells underneath the inner and outer hair cells. Morsli *et al.* (1998) suggested that *fringe* plays a similar role in the inner ear by specifying between sensory and nonsensory cells.

The mRNA expression of *notch1*, *jagged1* and *jagged2* in the cochlea has been determined by Lanford *et al.* (1998). At E13, *notch1* was distributed widely throughout the cochlear epithelium. *jagged1* was also expressed at E13 whilst *jagged* 2 was not expressed until E15. Their expression was localised to a narrow band that ran from the base to the apex of the cochlea. This band of expression migrated from the neural edge of the epithelium to the abneural edge. By P3, *jagged1* was restricted to supporting cells whilst *jagged2* was found only in hair cells. It would appear that the mechanism of lateral specification is critical for hair cell determination and depends, as it does in neural development, on the activity of Notch, its ligands and their modulators.

1.8 Hair cell differentiation

Once the fate of a sensory precursor cell within the epithelium has been determined it then undergoes differentiation. Initiation of differentiation in the cochlea begins at around E13 in the base and progresses towards the apex by E15 (Ruben, 1967; Nishida *et al.*, 1998). It occurs after terminal mitosis of the entire population of sensory precursor cells, which begins at E12 in the apex and finishes at around E14 in the base (Ruben 1967; Kelley *et al.*, 1993). One gene, *Brn3.1*, is critical for hair cell differentiation. It is a member of the POU-domain transcription factor family. This family of transcription factors was first found to play a critical role in neural development (Treacy and Rosenfeld, 1992; Wegner *et al.*, 1993). The POU-domain functions as a bipartite DNA-binding domain that contains a POU-specific domain of about 70 amino acids and a POU-homeodomain of about 60 amino acids, joined by a variable linker. It was first identified in the mammalian pituitary-specific Pit-1, the octamer binding proteins Oct-1 and Oct-2, and the *C.elegans* factor Unc-86 (Herr *et*

al., 1988). The POU-domain family is divided into four classes. The class III factor *Brn-4/POU3F4* is expressed in the rat otic vesicle (Le Moine and Young, 1992; Mathis *et al.*, 1992), and mutations in the corresponding human gene cause X-linked progressive sensorineural deafness and stapes fixation (DFN3) (de Kok *et al.*, 1995). The class IV factor *Brn3.1/ Brn3c/POU4F3* has been noted in sensory cell epithelium at E13 (Erkman *et al.*, 1996; Xiang *et al.*, 1997; Ryan, 1997; Xiang *et al.*, 1998). A null mutation of the gene leads to loss of only cochlear and vestibular hair cells, leading to profound deafness and vestibular dysfunction. In the human, a small deletion of the *Brn3.1* coding region has been linked to the autosomal dominant hearing loss DFNA15 (Vahava *et al.*, 1998).

Originally, it was thought that Brn3.1 was critical for the initial differentiation of the hair cell phenotype (Ryan, 1997). However, recent work by Xiang *et al.* (1998) has shown that in *Brn3.1*$^{-/-}$ mutants, hair cells undergo differentiation before degenerating via apoptosis. Their data indicates that *Brn3.1* is required for the maturation, survival and proper positioning of hair cells, whereas it appears to play virtually no role in the commitment and initial differentiation of hair cells. The downstream target of *Brn3.1* is an important factor to determine, and as yet there has been no conclusive evidence to suggest such a factor. However, *Brn3* genes are known to have a regulatory effect on the promoter of some acetylcholine genes (Milton *et al.*, 1996). They demonstrated that the regulatory region of the nicotinic acetylcholine receptor α2 subunit gene is activated by *Brn3.2* but not by *Brn3.0* or *Brn3.1.* Therefore, a good candidate for a downstream target of *Brn3.1* is the α9 acetycholine receptor subunit found in IHC and OHC (Elgoyhen *et al* 1994).

1.9 α9 acetycholine receptor subunit

The efferent innervation of the cochlea arises from neurons in the superior olivary complex nuclei (Rasmussen, 1946). Lateral olivocochlear neurons project to the ipsilateral cochlea and terminate on spiral ganglion cell dendrites under the IHCs. Medial olivocochlear neurons project to the contralateral cochlea and terminate on the bases of OHCs (Warr, 1992 for review). Several neurotransmitters are associated with efferent terminals including acetylcholine, γ-aminobutyric acid (GABA), enkephalin, dopamine, calcitonin gene related peptide (CGRP) and dynorphin (Hunter, 1992). Acetylcholine is the predominant neurotransmitter for both medial and lateral efferents (Housley and Ashmore, 1991; Eybalin, 1993).

Activation of the acetylcholine receptor (AChR) mediates a fast influx of Ca^{2+} into the OHC, which then activates an outwardly-directed Ca^{2+}-dependant K^+ channel (Sewell, 1997). The AChR is classified as a subunit of the neuronal nicotinic receptors with mixed nicotinic-muscarinic pharmacology (Housley *et al.*, 1992). Elgoyhen *et al.* (1994) isolated the subunit from the rat and named it α9. Recombinant DNA technology had already resulted in the identification of the vertebrate muscle nAChR subunits α1, β1, γ, δ, and ε, and the neuronal subunits α2, α3, α4, α5, α6, α7, α8, β2, β3, and β4 (Housley and Ryan, 1997).

α9nAChR mRNA has been found by *in situ* hybridisation and single cell reverse transcription-polymerase chain reaction in both OHC and IHC (Park *et al.*, 1997; Glowatzki, 1997). It first appears in the presumptive cochlear hair cells in the rat at E18 and peaks at P10 (Luo *et al.*, 1998). Its expression in IHC is not readily explained, as they do not receive direct efferent innervation. However, transient efferent innervation has been reported to occur during development (Lenoir *et al.*, 1980; Sobkowicz and Slapnick, 1994). For both IHC and OHC, the α9 protein is

concentrated at the base of the hair cell (Park *et al.*, 1997). The α9nAChR is specific for hair cells and can be used as a marker of hair cell differentiation.

1.10 Regeneration of hair cells

In mammals, cochlear hair cells are generated during embryogenesis, as described, and must remain viable throughout the lifetime of the organism to maintain normal auditory function. Critically, they are not replaced when they are damaged or die (Kelley *et al.*, 1995; Corwin and Oberholtzer, 1997; Forge *et al.*, 1998; Stone *et al.*, 1998). In contrast to mammals, continuous post-embryonic hair cell production occurs in fish, amphibians and birds (Corwin, 1985; Roberson *et al.*, 1992). Furthermore in birds, hair cells within the cochlea were regenerated following aminoglycoside and acoustic trauma (Cruz et al., 1987; Cotanche, 1987). The reinitiation of mitosis among the supporting cells within the sensory epithelium gives rise to new hair cells (Corwin and Cotanche, 1988; Ryals and Rubel 1988; Stone and Cotanche, 1994).

If it were feasible to regenerate or prevent degeneration of mammalian hair cells then an enormous amount of morbidity would be alleviated. This possibility was given added impetus by the discovery of hair cell replacement in the vestibular sensory epithelia of mature mammals (Forge *et al.*, 1993; Warchol *et al.*, 1993). Forge *et al.* demonstrated the appearance of immature stereociliary bundles *in situ* in the vestibular epithelia of adult guinea pigs, which increased in number several weeks after aminoglycoside induced hair cell loss. Complementary studies performed *in vitro* with organotypic explants of utricular macula from mature guinea pigs suggested that sensory epithelial cells can be induced to divide following aminoglycoside induced damage (Warchol *et al.*, 1993). However, the exact mechanism by which the adult hair cells are replaced or repaired remains elusive.

In non-mammalian species, hair cells have arisen directly from supporting cells without an intervening mitotic event (Baird et al., 1993; Baird et al., 1996; Roberson et al., 1996; Adler et al., 1997; Steyger et al., 1997). In mice, Kelley *et al.* (1995) found that embryonic cochlear hair cells, and on rare occasions neonatal hair cells, were replaced after laser microbeam irradiation. They suggested that supporting cells were able to change their pre-existing fate in response to hair cell damage. This supports the concept of lateral specification, for as the supporting cells are released from this mechanism they become hair cells. In the adult guinea pig, Forge *et al.* (1998) showed that spontaneous limited recovery did occur in vestibular hair cells following aminoglycoside damage. They, too, propose that the new hair cells were derived, without having to undergo mitosis, from existing supporting cells.

Evidence supporting the role of repair in hair cells that have been subjected to sub-lethal damage, has come from work on the developing organ of Corti *in vitro* (Sobkowicz *et al.*, 1997). Furthermore, microvillar tufts resembling immature stereociliary bundles were detected in young rats after drug induced damage (Romand *et al.*, 1996; Lenoir *et al.*, 1997), suggesting that cochlear hair cells do attempt to regenerate but fail to differentiate and eventually die.

1.11 Cochlear preparations

In order to study the current complexities surrounding hair cell regeneration, recovery, repair and maintenance as well as the normal development of hair cells it is necessary to have a suitable cochlear preparation. For many years tissue cultures of the organ of Corti have been used to study the development of the cochlea

(Sobkowicz *et al.*, 1993). In particular, studies of the ultrastructural development of the sensory and supporting elements of the inner ear, the development and maintenance of the afferent innervation, and the regeneration of the organ of Corti, have all been analysed using tissue culture (Sobkowicz *et al.*, 1975; Sobkowicz *et al.*, 1984; Sobkowicz *et al.*, 1992). However, one of the major disadvantages associated with organ tissue culture is that the cells do not survive for longer than a couple of weeks. Moreover, they have only limited potential for analysing gene function. The more traditional method of identifying genes and understanding their function has been the use of randomly generated mutations. In the mouse, many mutants are known, such as *kreisler* (Cordes and Barsh, 1994), that show an ear phenotype and represent good models for human deafness (Petit, 1996). The zebrafish mutants have also become a rich source for identifying novel genes in ear development (Whitfield *et al.*, 1997). Other methods used to find new genes include subtractive hybridisation (Robertson *et al.*, 1994), gene trapping (Yang *et al.*, 1998) and the use of specific probes to isolate the gene encoding a particular protein. More recently, with progress in the genome project there is an opportunity to isolate a large number of genes responsible for normal cochlear function and deafness using a scanning genome array (Chen *et al.*, 1998). However, without a suitable *in vitro* system to explore and characterise these genes more easily then the pace of subsequent progress will be slow. Furthermore, the relative inaccessibility of the cochlea and the small number of hair cells do hamper any research into the molecular genetics of the inner ear.

The need for an *in vitro* system that has the advantages of tissue culture and the ability to fulfil the dual entities of identification and functional analysis of genes is paramount. Cell lines offer the opportunity to study the mechanisms of hair cell determination and differentiation and importantly they will provide an almost

unlimited source for the biochemical isolation of low abundance molecules. That such a system is theoretically possible comes from work performed on other cell types. The production of cell lines that have been genetically manipulated to create an immortal and, importantly, stable culture of cells has made such a scenario possible.

1.12 Production of cell lines

In tissue culture, differentiated somatic cells have a limited life span. They divide a finite number of times before they arrest (senesce). Even if they are given forced signals, eg. growth factors, to undergo continued division they will still eventually die (crisis). The current model to explain this phenomenon implicates the critical role of telomerase in this process of ageing (Greider, 1994). As telomeres shorten with each successive round of mitosis, at some critical length, a genetic program of senescence is switched on. By introducing specific viral oncogenes into dividing mortal cells, cellular programs for limiting the rate and the number of replications are inhibited and the proteins involved in senescence are inactivated (Hopfer *et al.*, 1996). The cells are now deemed to be immortal. It is important that the transfected cell line retains the differentiated characteristics of the mortal cell if it is to have any benefit as an experimental model. This has proven to be the case in immortalised cell lines used to study cystic fibrosis (Gruenert *et al.*, 1995).

Recently, several viral oncogenes have been identified which increase the probability of successful immortalisation of primary tissue culture cells. These include; simian virus 40 (SV40) large T antigen (TAg), polyoma TAg, human papillomavirus E6 and E7 and adenovirus E1a (Rassoulzadegan *et al.*, 1982; Jat *et al.*, 1986). The ideal animal to facilitate cell line generation would be one where the immortalising gene is inactive *in vivo* and does not impair the health of the animal but can be conditionally

activated by the investigator, ie., in tissue culture. Conditional activation is an important feature not only as a means of preventing tumour formation but also as a means of regulating experimental conditions. If the activation mechanism were generally applicable to all cell types, then a wide variety of cell lines could be derived which had similar expression of the oncogene. The oncogene could then be switched off *in vitro* so that differentiation of the tissue could be studied.

The $H2k^b$*ts*A58 transgenic mouse (Immortomouse) fufills all these criteria (Jat *et al.*, 1991). It contains a temperature sensitive immortalizing oncogene (*ts*A58) derived from a mutant SV40 and can be used to establish cell lines from the most challenging material. The ubiquitous major histocompatibility complex $H2k^b$ class-1 promoter controls its expression, which can be upregulated in the prescence of γ-interferon (γIFN) (Kimura *et al.*, 1986; Baldwin *et al.*, 1987). The gene codes for a thermolabile variant of TAg that is stable at 33°C but is rapidly degraded *in vivo* at mouse body temperature (37°C-40°C) (Jat *et al.*, 1989). Thus every cell within the mouse should possess a copy of an immortalising gene which can be conditionally expressed by the control of both temperature and γIFN. The health of the animals is not impaired, as the oncogene is inactive at body temperature. So far the only abnormality noted is hyperplasia of the thymus (Jat *et al.*, 1991). Experimentally, *in vitro,* the cells proliferate at 33°C when TAg is stabilised and upregulated by γIFN. But when the TAg is switched off at 39°C in the absence of γIFN the cells subsequently differentiate (Fig.1.5). The oncogene perpetuates cell division and prevents terminal differentiation by abrogating both the p53 and retinoblastoma tumor suppressor pathways (Parkinson, 1996). The Immortomouse has been used widely in other fields to investigate cells *in vitro.* These include rat embryo fibroblasts (Gonos *et al.*, 1996), striatal cells from the central nervous system (Giordano *et al.*, 1996), bovine

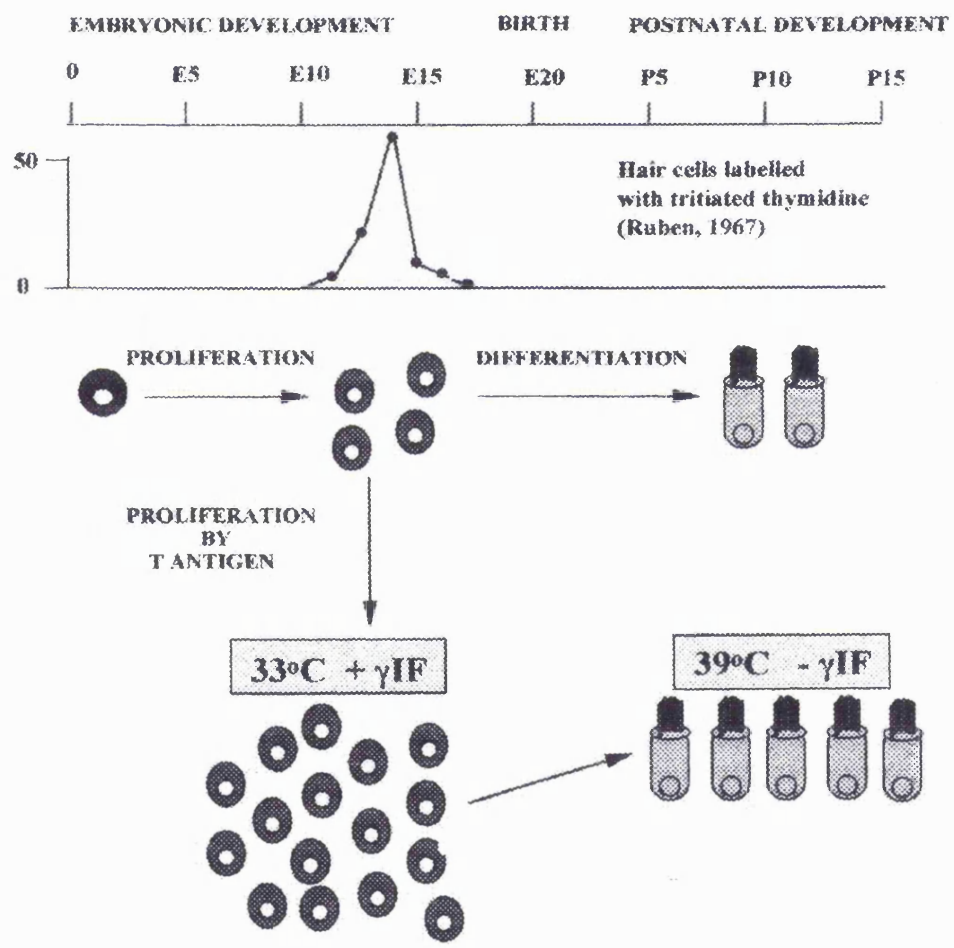
mammary epithelia (Zavizion *et al.*, 1996), glial cells (Bernard *et al.*, 1994), cells from the otocyst (Barald *et al.*, 1997) and vestibular epithelial cells (Holley *et al.*, 1997).

Mammalian cochlear hair cells undergo terminal mitosis by embryonic day 14 (E14) prior to terminal differentiation (Ruben, 1967). The cell line UB/OC-1 (University - Bristol/Organ of Corti-1) was established from epithelial cells derived from the embryonic cochlea at E13, thus providing the first known established *in vitro* embryonic cochlear hair cell lines (UB/OC-1 and UB/OC-2) (Rivolta *et al.*, 1998).

E13 organ of Corti cultures from the Immortomouse were grown at 33°C in the presence of γIFN. The cultures were then dissociated after the initial population expansion and the process of selective adhesion was used to enrich the epithelial cells. The epithelial cells were then cloned by limiting dilution to produce homogeneous (UB/OC-2) and heterogeneous (UB/OC-1) cell lines (Grix, PhD thesis, 1999). UB/OC-1 was characterised and found to contain exclusively hair cells which expressed *Brn 3.1, α9AChR, Myo7a,* and fimbrin at 39°C (Rivolta *et al.*, 1998). At 33°C, there were trace amounts of *Brn3.1, Myo7a,* fimbrin but no *α9AChR.* Electrophysiological studies showed that the α9AChR was functionally active in 30% of the cells at 39°C.

FIGURE.1.5

Experimental Design. Cell lines were derived prior to terminal mitosis and the TAg was switched on at 33°C in the prescence of γIFN. TAg was switched off at 39°C in the absence of γIFN allowing differentiation to occur. At 33°C cells were harvested after 2 days for all experimental procedures (equivalent to day 0). At 39°C cells were harvested after 14 days for all experimental procedures unless otherwise specified.



1.13 Aims and outline of the present study

The purpose of this study was to examine a number of features of cochlear hair cell fate determination and differentiation. By looking at the pattern of expression of some of the genes involved in these processes it was anticipated that a model could be proposed that might explain our current understanding of the mechanism of lateral specification. This knowledge is important to our understanding of the wider issues of hair cell survival and regeneration. The long-term goal will always be the cure and prevention of sensorineural deafness.

The cell line UB/OC-1 could provide an ideal experimental tool with which to explore some of these fundamental issues. To establish this important role it not only has to provide a viable *in vitro* system but it also must be able to relate to hair cell development *in vivo*.

The study has been divided into four separate experiments. At the time of the first two experiments the evidence suggested that *Brn3.1* was the critical factor necessary for the initial stage of hair cell differentiation. Indeed, it had been postulated that *Brn3.1* was the factor involved in hair cell commitment. Furthermore, there was little information as to what might be the downstream effects of *Brn3.1*. There was the possibility of a causal link between the expression of *Brn3.1* and the *α9AChR*.

The first experiment involved looking at the expression of *Brn3.1* and the *α9AChR* at selected time points under differentiating conditions. Particularly, this would establish whether or not there was the potential for a link between the two genes. It would also provide some information about whether the timing and sequence of expression of these genes in this *in vitro* system is similar to that of normal development and thereby provide verification that the cell line is a potentially useful experimental tool.

Secondly, the aim was to derive clonal cell lines from UB/OC-1, which would not express *Brn3.1* under proliferating conditions. This would then provide the opportunity to alter the fate of the cells and convert them to supporting cells. It would also provide an opportunity to study the early differentiation of hair cells.

The remaining two sets of experiments involved the characterisation of a selected subset of clones derived from UB/OC-1. The hypotheses were:

1. Would the clones continue to express epithelial and hair cell specific characteristics under differentiating conditions and if so would this provide information as to the differentiation of hair cells?
2. Would the clones express the receptors and ligands involved in lateral specification in a manner consistent with that of normal development and could the pattern of expression help in forming a model that could explain the mechanism involved in hair cell fate determination?

The principle methods of investigation employed for these experiments were cell culture, immunocytochemistry, reverse transcription polymerase chain reaction (RT-PCR) and western blot.

CHAPTER TWO

METHODS

2.1 Cell Culture.

The cell line UB/OC-1 and its sub-clones were cultured in Minimal Essential Medium (MEM) with Earle's salts and Glutamax (Gibco BRL), and 10% fetal calf serum (FCS) (Gibco BRL) in a 75cm^2 polystyrene tissue cultured flask (Corning). Under proliferating conditions at 33°C, 50U/ml γ-interferon (γIFN) (Sigma) was added. When the cells reached confluence they were washed with modified Hanks Balanced Salt Solution (HBSS) (Sigma) containing 10mM Hepes (Sigma) and trypsinized with 2.5mg/ml trypsin (Sigma) diluted in HBSS. When the cells had rounded up the trypsin was inactivated with an equal volume of culture medium. The suspension was centrifuged at 180g for 3 minutes and the pellet was then resuspended in 5ml of medium. 10μl aliquots were placed in a haemocytometer (Weber) and the cells counted. The cell cultures were split as appropriate. Cells for storage were frozen at a concentration of 5x10^5 cells in 0.5ml of 10% dimethyl sulphoxide (DMSO) (Sigma) and 90% FCS and stored in liquid nitrogen. For reculturing, the cells were thawed rapidly at 37^{0}C for 3 minutes, washed with MEM, centrifuged at 180g for 5 minutes and resuspended in culture medium. Under differentiating conditions at 39^{0}C cells were cultured in MEM and 10% FCS in the absence of γIFN. (From now on "33^{0}C" means 33^{0}C and γIFN, whilst "39^{0}C" means 39^{0}C in the absence of γIFN). At both 33^{0}C and 39^{0}C the cells were fed twice weekly and incubated in 5% CO_2 (RSBiotech incubators).

Sub-clones were derived by limiting dilution (Freshney 1994) from single cell suspensions plated out in two flat bottomed 96 well plates (Costar). For each plate the

cell suspension was diluted to obtain 96 cells in 10ml medium and 100µl of the diluted suspension was added to each well (1cell/well). Plates were incubated at 33^0C for 3 weeks and fed with 50µl of 50% filtered conditioned medium from UB/OC-1 at 33°C and 50% normal medium until they had reached confluence. 24 wells produced viable cells which were then trypsinized and placed in two 12 well plates (Costar) and subsequently into 75cm^2 flasks.

For immunolabelling and RT-PCR cells were seeded at a density of 83/mm^2 at 33°C in a 35mm dish (Falcon) and 104-208/mm^2 in a 35mm diameter dish at 39°C. For Western Blot cells were seeded at a density of 6667/cm^2 at 33°C and 13,333/cm^2 at 39°C in 75cm^2 flasks.

2.2 Immunocytochemistry.

Cultures were rinsed in MEM and fixed for 10 minutes with either 4% paraformaldehyde in phosphate buffered saline (PBS) (pH7.5), or 1:1 Acetone/Methanol depending on the antibody used. Paraformaldehyde-fixed cells were rinsed with Tris-Buffered Saline (TBS) (137mM NaCl, 2mM KCl, 11.5mM Tris, 38mM Trizma, 3mM Sodium Azide, pH 7.6) and permeabilized with 0.5% Triton X-100 (Sigma), whilst the acetone/methanol cells were air dried for 20 minutes. A blocking solution of 5% goat serum in TBS was used for 20 minutes before the primary antibody was added. The primary antibodies were as follows:- Brn3.1 antibody (dilution 1:250) is a rabbit polyclonal (Xiang *et al* 1995), obtained from BAbCo, California. The monoclonal anti-pan cytokeratin antibody (dilution 1:50), the polyclonal anti-pan cadherin antibody (dilution 1:50), and the monoclonal anti-vimentin antibody (dilution 1:200) were from Sigma (C-2562, C-3678 and V-5255 respectively). F-actin was labelled with Texas-Red–X phalloidin (dilution 1:40)

(Molecular Probes). Mouse ascites, rabbit serum or TBS were used as the appropriate controls. The primary antibodies were left on overnight at 4°C. Brn 3.1 was visualised with an Enzyme-Labelled Fluorescence Amplification kit (ELF-AP) (Molecular Probes, Oregon). This utilises an alkaline phosphatase substrate that yields a fluorescent precipitate. The biotinylated alkaline phosphatase is linked by streptavidin to the biotinylated secondary antibody ensuring adequate amplification of the signal allowing detection of low abundance molecules. Cytokeratin, cadherin, and vimentin were visualised with an FITC-conjugated secondary antibody (dilution 1:200). The secondary antibody was washed with TBS before 4,6-Diamidino-2-phenylindole (DAPI) (dilution 1μg/ml) was added to label the nuclei. One final wash with TBS was performed before mounting with Vectashield (Vector Laboratories) and a 32mm glass coverslip. Phase contrast pictures were taken on an inverted DM IRB Leica microscope using Ilford FP4 125 black and white film. Immunofluoresence pictures were taken using a Nikon Optiphot-2 microscope using Kodak Tmax 400 black and white film or Agfa RSX 200 colour film. The negatives were developed commercially. The black and white pictures were printed on Multigrade IV RC de luxe paper (Ilford) from the negatives using a Durst Laborator 1000 enlarger, 1:9 solution of Ilford Phenisol film developer and 1:9 solution of Ilford Hypam fixer.

2.3 Reverse Transcription Polymerase Chain Reaction.

Total RNA was extracted from UB/OC-1 and the clones at 33°C and 39°C using RNeasy preparation (Qiagen). The concentration of RNA was determined using a spectrophotometer. 2μg of total RNA was used to synthesise cDNA. Genomic DNA contamination was removed by incubating the samples with 1.5U of RQ1 DNase enzyme (Promega) in the presence of 40U RNase Block Ribonuclease Inhibitor

(Stratagene) in buffer of 40mM Tris, 8mM $MgCl_2$, 50mM NaCl (pH8) at 37°C for 15 minutes. RQ1 DNase was inactivated by a 1:1 phenol/chloroform extraction and then the RNA was ethanol precipitated. For every 50µl reaction of cDNA synthesis, 300ng OligodT was needed to anneal to the RNA at 65°C for 5 minutes. This took place before reverse transcription occurred at 37°C for 90 minutes in the presence of 4mM dNTPs, 40U RNasin, Moloney Murine Leukaemia Virus (MMLV) buffer (50mM Tris-HCl, 75mM KCl, 3mM $MgCl_2$, pH8.3) and 50U MMLV-RT (Stratagene). The cDNA was incubated at 90°C for 5 minutes to inactivate the MMLV and then stored at -80°C.

Primers (Gibco) employed for the detection of the different transcripts correspond to mouse sequences whilst that of *α9* comes from the rat. *gapdh* is a common 'house keeping' gene which is used as a normalising control. The primers were: *gapdh*, position 248 (5' AACGGGAAGCCCATCACC 3') and 672 (5' CAGCCTTGGCAGCACCAG 3'); *Brn3.1* (A) position 205 (5' CCATGCGCCGACTTTGTCTCC 3') and 639 (5' CTCCACATCGCTGAGACACGC 3') and (B) position 444 (5' GCGGTACATCAGGGCCTC 3') and 656 (5' CACATCGCTGAGACACGC 3'); *α9*, position 754 (5' CCTTACCCAGATGTCACCTTCACTC 3') and 1466 (5' AACACCATAGCAAAGAAAATCCACA 3'); *Myo 7a*, positions 468 (5' GCTGTATTATCTGCGGGGAG 3') and 856 (5' CTGGTGATGCACTTCCCCATG 3'); *Jag2* (Forward/Upper 5' TGCATGGATGGCTGGATG 3') and (Reverse/Lower 5'GTGCTGTGATGGAGGGGTCA 3'). *Jag2* sequences were kindly supplied by Dr.Matthew Kelly, Georgetown University, Washington DC, USA. PCR reactions were set up according to a standardised protocol (Innis *et al.*, 1990). Different concentrations of buffer, magnesium, dNTP, primers and *Thermus aquaticus* DNA

Polymerase (*Taq* DNA Polymerase) were used (Fig.2.1). An equal amount of cDNA was used for each reaction (3µl in a final volume of 50µl). Mineral oil (Sigma) was added to each reaction to prevent evaporation. The PCR thermal cycles of "hot start", denaturing, annealing, extension, final extension and cycle length were determined according to the length of the gene product (Fig.2.2). A "hot start" was employed to allow complete denaturation of the cDNA thereby preventing any false priming. PCR's were performed under conditions that maintained the amplifications within the comparable, exponential phase determined by previous kinetic analysis. dH_2O was used in every reaction as a none template control to ensure that amplification was from the template and not from any contamination. The identities of the PCR products for *Brn3.1*, *α9* and *myo7a* were confirmed by sequencing analysis and restriction enzyme digestion.

GENE	**10x BUFFER**	**Mg (mM)**	**dNTP (mM)**	**UPPER (pM)**	**LOWER (µM)**	**Taq (U)**
Brn3.1 (A)	5µl	2	0.2	50	50	2.5
Brn3.1 (B)	5µl	1.5	0.2	50	50	2.5
α9	5µl	2	0.2	50	50	2.5
Myo 7a	5µl	2	0.2	50	50	2.5
Jag 2	5µl	2	0.2	50	50	2.5
gapdh	5µl	2	0.2	50	50	1.25

FIGURE.2.1. Values are for each 50µl reaction. Final volumes are made up with dH_2O. 10x Reaction Buffer; 500mM KCl, 100mM Tris HCl (pH 9.0 at 25°C), 1.0% Triton X-100. 25mM $MgCl_2$. 0.2mM dNTP. 5U/µl *Taq* DNA Polymerase stored in 50mM Tris-HCl (pH 8), 100mM NaCl, 0.1mM EDTA, 5mM DTT, 50% glycerol and 1% Triton X-100. All reagents supplied by Promega.

GENE	Hot Start (Temp/min)	Denaturing (Temp/sec)	Annealing (Temp/sec)	Extension (Temp/sec)	Final Extension (Temp/min)	Number of cycles
Brn3.1 A	94°C/5 min	94°C/15s	60°C/15s	72°C/40s	72°C/1 min	40
Brn3.1 B	94°C/5 min	94°C/15s	64°C/15s	72°C/40s	72°C/1 min	40
α9	94°C/5 min	94°C/15s	60°C/15s	72°C/40s	72°C/3 min	40
Myo 7a	94°C/5 min	94°C/15s	60°C/15s	72°C/30s	72°C/1 min	35
Jag 2	94°C/5 min	94°C/15s	58°C/15s	72°C/30s	72°C/7 min	28
gapdh	94°C/5 min	94°C/15s	60°C/15s	72°C/40s	na	25

FIGURE.2.2 PCR reactions were performed in a GeneAmp PCR System 2400 (Perkin Elmer). *gapdh* was used as a control for each PCR reaction and removed after 25 cycles.

20μl of the PCR product was combined with 5μl of cDNA loading buffer and run at a constant 70 volts through a 1.5% agarose gel (40mM Tris-Acetate, 1mM ethylenediaminetetraacetic acid (TAE) buffer, 1.5% Agarose Molecular GQT Grade and 1μg/ml ethidium bromide), in TAE buffer solution containing 1μg/ml ethidium bromide. A 1kb DNA ladder (GeneRuler, 0.5mgDNA/ml. MBI Fermenta) was used to determine the length of the PCR product. Photographs were taken under a UVP Dual-Intensity Transilluminator with 667 Polaroid film. Images were edited using Adobe Photoshop.

2.4 Western Blot

Protein extraction from the cells was performed on ice to minimise protease activity. Cells were washed with MEM and PBS before extraction with buffer (150mM NaCl, 1%NP-40, 0.1% Sodium dodecyl sulphate (SDS), 50mM Tris (pH 7.4)). The cells were homogenised and then ultra-centrifuged for 10 minutes at 10,000g and 4°C. For the protein assay, 1ml of bicinchoninic acid (BCA) Reagent (50ml Reagent A and 1ml Reagent B, Pierce) was mixed with 50µl aliquots from known concentrations of bovine serum albumin (2000µg/ml, 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml), and a 1 and 1:5 dilution of the cell samples. All samples were incubated at 37°C for 30 minutes. The absorption was read in a double beam spectrophotometer set at a wavelength of 562nm. Sample protein concentration was interpolated from the standard curve. Protein extract was stored at -80°C in a reducing SDS sample buffer containing bromophenol blue.

A SDS-Polyacrylamide gel electrophoresis system (Laemmli, 1970) was used to determine the molecular weights of the polypeptides. Standard proteins of known molecular weight (BioRad) were run along side the polypeptide to be characterised. The treated peptides were first stacked in a stacking gel before entering the separating gel. Different separating gels were used depending on the molecular weight of the peptide; 7.5% gel for high molecular weight peptides, a 10 % gel for low molecular weight peptides and a 14% gel for very low weight peptides. The separating gel was made from stock solutions of 30%T 2.7%C_{bis} Monomer solution, 1.5M Tris-HCl (pH 8.8) buffer, 10% SDS, 10% Ammonium Persulphate and 0.5% *N,N,N',N'*-tetramethylenediamine (TEMED). The gel was covered with water-saturated n-butanol and allowed to set. Overlay buffer (0.375M Tris-HCl, 0.1% SDS, pH8.8) was

applied before the stacking gel was added, which was made from stock solutions of 30%T 2.7%C_{bis} Monomer solution, 0.5M Tris-HCl (pH 6.8) buffer, 10% SDS, 10% Ammonium Persulphate and 1% TEMED. 5μg of protein from each cell extract was loaded and run in an electrophoresis buffer of 0.025M Tris, 0.192M glycine, 0.1%SDS, (pH 8.3) at 20mA.

Gels were transferred to nitrocellulose membranes (Sartorius) in the presence of transfer buffer (25mM Tris, 192mM glycine, 5% methanol). High molecular weight polypeptides (MyosinVIIA) required 20% methanol and 0.1% SDS (Bolt and Mahoney, 1997) for appropriate transfer. In all cases, a semi dry transfer method was used for 90 minutes at 40mA in a LKG 2117 Multiphor II Electrophoresis Unit (Pharmacia).

After blotting the membranes were immersed overnight at 4°C in PBS containing 2.5% bovine serum albumin (BSA) and 2.5% non-fat dry milk mixture. The standard was washed with PBS and placed in AuroDye (Amersham) overnight. The primary antibodies used were; Brn 3.1 (dilution 1:400); Fimbrin (dilution 1:1500, a gift from Dr.P.Matsudaira, Massachusetts Institute for Technology, Cambridge, USA); MyosinVIIa (dilution 1:1000, a gift from Dr.C.Petit, Pasteur Institute, Paris), (El-Amraoui *et al* 1996); Jagged 1 (dilution 1:200) (Santa Cruz Biotechnology), Notch 1 (dilution 1:200) (Santa Cruz Biotechnology), Numblike (dilution 1: 1000, a gift from Dr.Y.Jan, Howard Hughes Medical Institute, University of California, San Fransisco); OCP-2 (dilution 1:1000, a gift from Professor.R.Thalmann, Washington University, St Louis, USA). The primary antibody was diluted in blocking solution and left on the membrane for 3 hours. The membrane was then washed with PBS and 0.05% $Tween^{20}$ before the secondary antibody was added. The secondary antibodies were peroxidase-conjugated monoclonal anti-goat/sheep, anti-rabbit or anti-rat (dilution

1:5000). They were left on for one hour and then the membrane was washed again. SuperSignal chemiluminescent substrate (Pierce) was used to activate the peroxidase for 5 minutes. The membrane was loaded in a Hypercassette (Amersham) with Hyperfilm-ECL (Amersham) for a variable period of time. The film was processed using 20% solution of Ilford Phenisol developer and a 20% solution of Ilford Hypam fixer. Images were scanned into IBM desktop computer and edited using Adobe Photoshop.

CHAPTER THREE

RESULTS

3.1 Temporal expression of *Brn3.1* and *α9* in UB/OC-1.

UB/OC-1 (passage 19 [P.19]) was grown in culture at 33°C and 39°C. The passage number refers to the number of times the cell line has been trypsinised. mRNA was collected at 33°C (day 0) and at time points 3, 6, 9, 16, 23 and 30 days at 39°C. This was repeated on a separate occasion with cells at the same passage number.

3.1.1 Expression of *Brn3.1*.

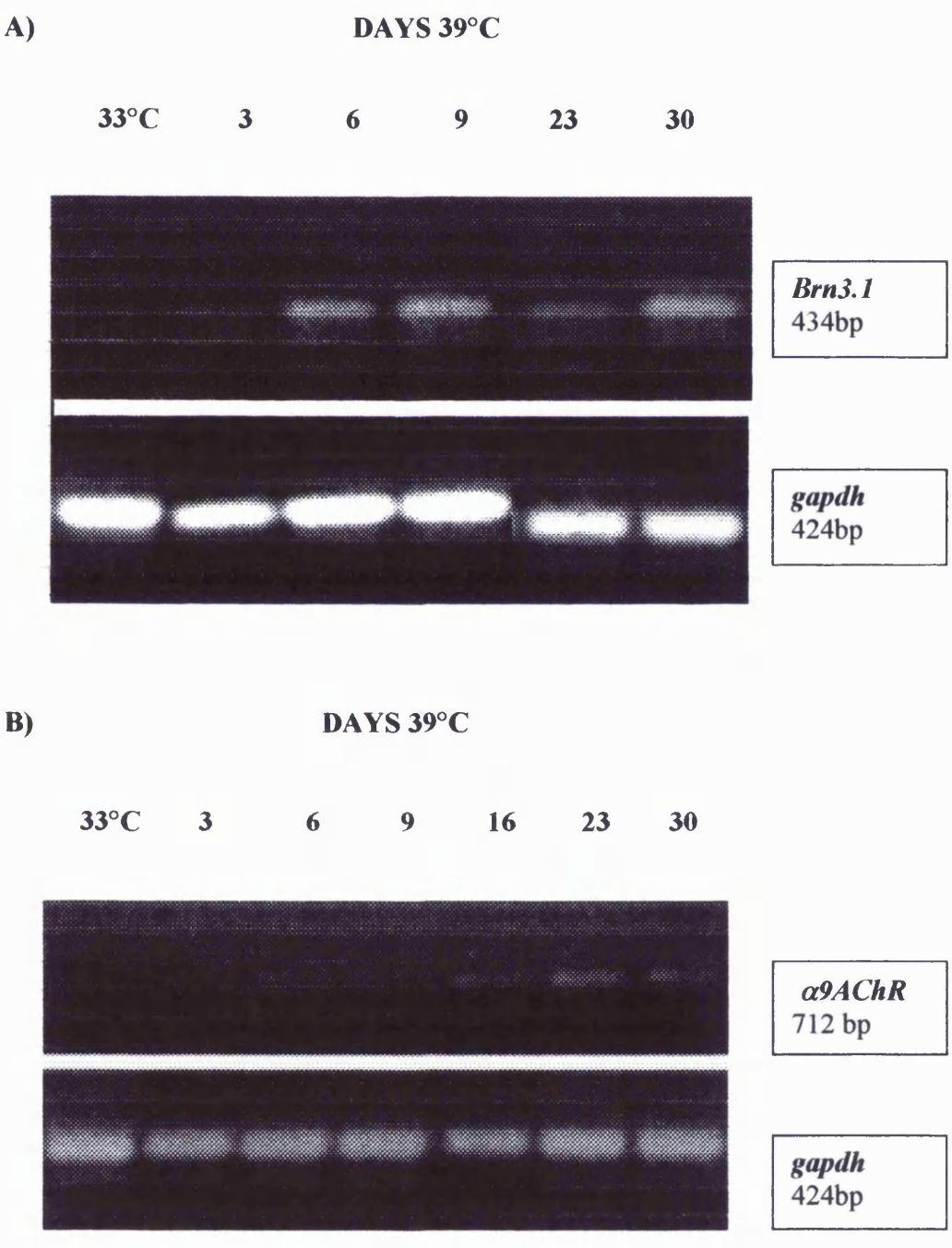
Results from the RT-PCR show that *Brn3.1* was barely detectable at 33°C but at 39°C it appeared to be upregulated by day 3 and continued to be expressed at day 30. (Fig.3.1a).

3.1.2 Expression of *α9*

Analysis of the *α9AChR* mRNA by RT-PCR showed no expression at 33°C. It was first detected after 3-6 days at 39°C and was present throughout the full 30 day period (Fig.3.1b).

FIGURE.3.1

RT-PCR of *Brn3.1* and *α9AChR* over the time course. (Day 0 corresponds to 33°C).



3.2 Cloning UB/OC-1 to test for clonality and generate *Brn 3.1* negative cell lines

Results from the previous experiment showed that there were very low levels of *Brn3.1* expression at 33°C. UB/OC-1 (P.10) was recloned to determine whether the low level of expression was uniformly distributed through out all the cells or whether a just some cells were expressing it.

24 clones were derived from the original 196 wells (12.2%). 10 of the clones became infected and were discarded. The remaining 14 clones were labelled with polyclonal Brn3.1 antibody at 33°C and 39°C. The primary antibody (Brn3.1) was omitted and replaced by normal rabbit serum in the controls. Six random cell counts using a microscope counting grid were performed under the same magnification (x200) for each clone at each temperature to determine the number of Brn3.1 positive and negative cells (Fig.3.2).

At 33°C, 7 clones appeared negative, 6 were indeterminate due to background labelling and 2 had positive labelling for Brn3.1 (#21 and #24). At 39°C, all clones except #1 expressed Brn3.1 in all cells. On the basis of these results clones #14, #15 (Brn3.1 negative at 33°C) and #24 (Brn3.1 positive at 33°C) (Figs.3.3 & 3.4) were selected for further characterisation.

UB/OC-1 was derived from a dilution equivalent to 10cells/well (Grix PhD thesis, 1999). 43% of the wells from her 96 well plate resulted in a population of cells. 12% of the wells from the above cloning experiment resulted in a population of cells. The probability that #14, #15 and #24 are truly representative of a homogenous clonal population is 99% (Coller and Coller, 1986).

The population doubling times (PDT) at 33°C were determined for the three subclones using the equation: PDT= $1/r$. Where $r=3.32(\log N_H-\log N_I)/(t_2-t_1)$

N_H=number of cells harvested at the end of the growth period. N_I=number of cells seeded. t_2=number of hours cells have been in culture (5 days). t_1=time zero.

CLONE	33°C Labelled. Brn3.1 positive cells (%)	33°C Control. Brn3.1 positive cells (%)	39°C Labelled. Brn3.1 positive cells (%)	39°C Control. Brn3.1 positive cells (%)
1	0 n=426	0 n=252	85(15) n=78	0 n=60
2	0(22) n=222	0 n=112	100 n=192	0 n=87
7	0 n=1542	0 n=984	100 n=198	0 n=36
14	0 n=288	0 n=184	100 n=486	0 n=564
15	0 n=534	0 n=408	100 n=402	0 n=224
16	0(18.5) n=324	0 n=136	100 n=264	0 n=144
17	0(9) n=192	0 n=124	100 n=348	0 n=204
18	0 n=360	0 n=328	100 n=522	0 n=308
19	0 n=438	0 n=176	100 n=258	0 n=268
20	0(28) n=125	0 n=148	100 n=246	0 n=116
21	59(15) n=204	0 n=168	100 n=174	0 n=68
22	0 n=306	0 n=172	100 n=168	0 n=208
23	0(13) n=228	0 n=168	100 n=270	0 n=100
24	92 n=396	0 n=148	100 n=210	0 n=164

FIGURE.3.2

Table of clones labelled with Brn3.1. Figures in brackets () represent percentage of cells where the labelling was indeterminate and there was possible but not definite positive labelling. n= the total number of cells counted.

FIGURE.3.3

Phase contrast pictures taken of clones #14, #15 and #24 at 33°C and 39°C. (x200).

A.

#14/33P.20. The cells are rapidly proliferating and have a "fibroblastic like" morphology. Average PDT=29 hours per doubling.

B.

#14/39P.19 after 14 days in culture. The cells have adopted a more epithelial like morphology appearing more symmetrical and assuming a mosaic like pattern.

C.

#15/33P.19. The cells are rapidly proliferating and have a "fibroblastic like" morphology. Average PDT=29 hours per doubling.

D.

#15/39P.18 after 14 days in culture. The cells have adopted a more epithelial like morphology appearing more symmetrical and assuming a mosaic like pattern.

E.

#24/33P.19. The cells appear more epithelial like. Average PDT= 42 hours per doubling.

F.

#24/39P.18 after 14 days in culture. The cells continue to adopt an epithelial like morphology and mosaic pattern.

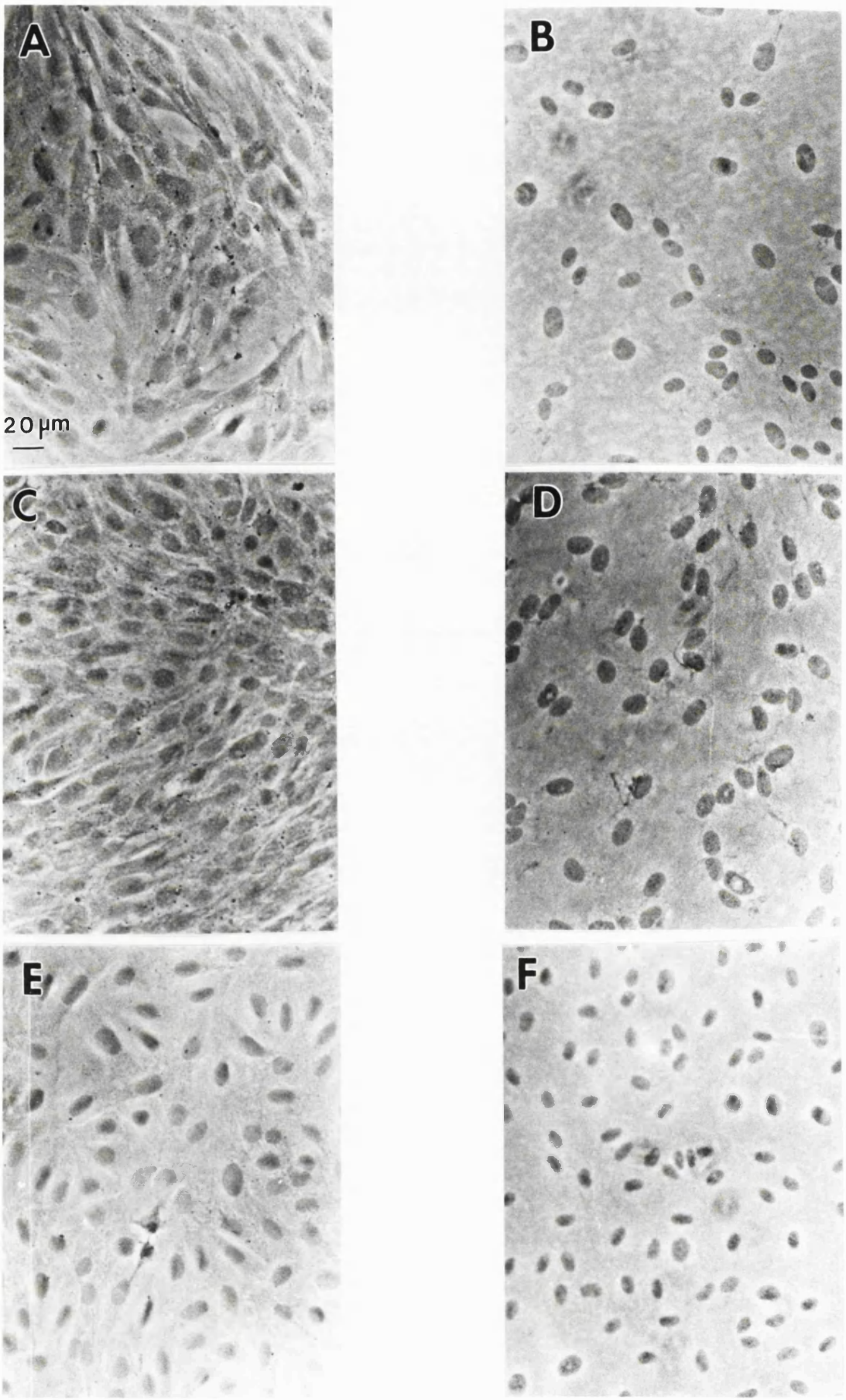

A
20 µm
B
C
D
E
F

FIGURE.3.4

Brn3.1 immunolabelling of #14, #15 and #24 at 33°C and 39°C. (x200).

33°C

A. Brn3.1 Antibody

#14/33P.15. No nuclei appear to be labelled.

B. Control

#14/33P.15. No nuclei appear to be labelled.

C. Brn3.1 Antibody

#15/33P.15. No nuclei appear to be labelled although there is marked background labelling.

D. Control

#15/33P.15. No nuclei appear to be labelled.

E. Brn3.1 Antibody

#24/33P.14. Nuclei are labelled with Brn3.1 antibody.

F. Control

#24/33P.14. No nuclei appear to be labelled.

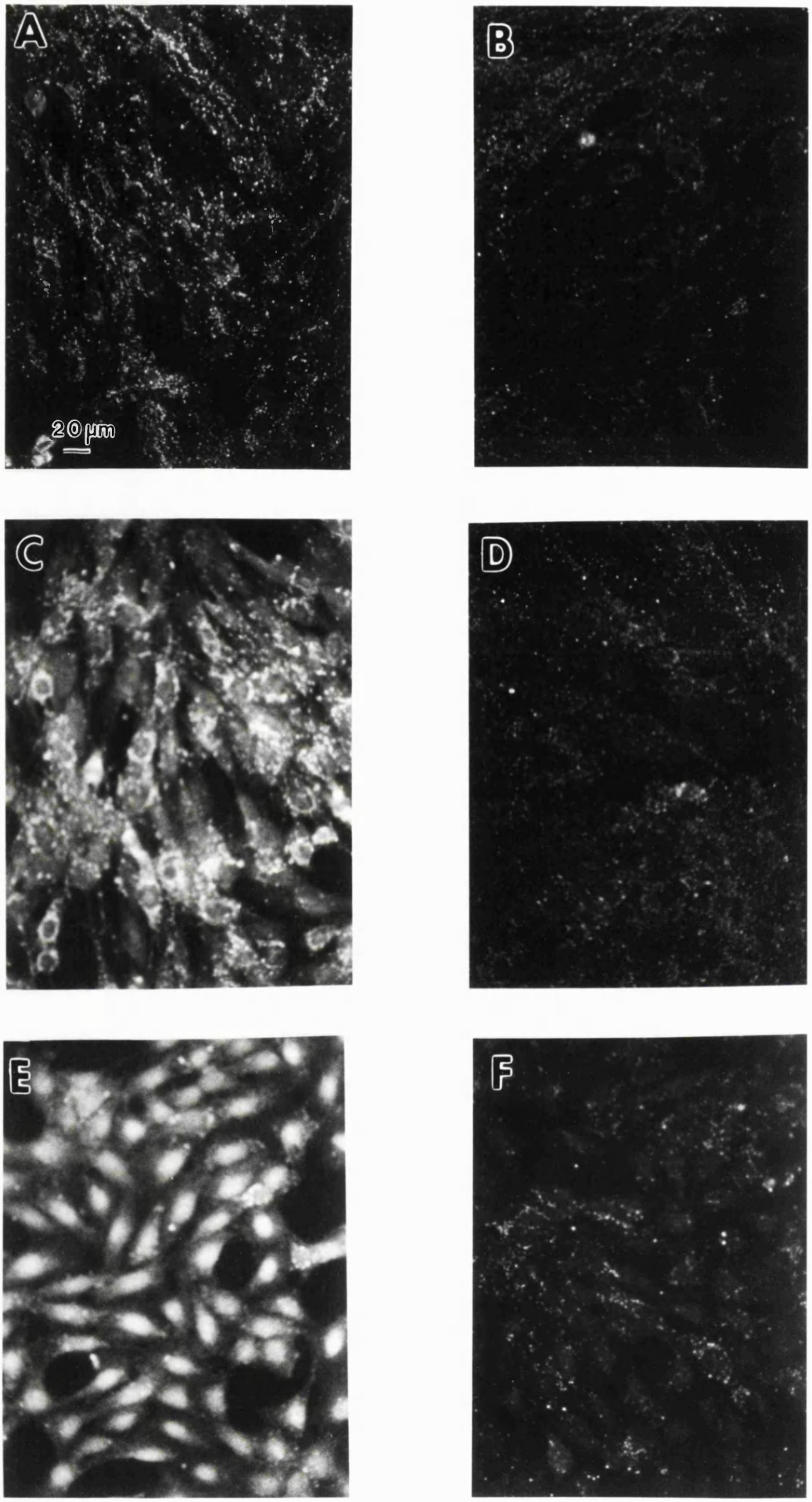

A
20 µm
B
C
D
E
F

FIGURE.3.4 continued.

39°C

G. Brn3.1 Antibody

#14/39P.14. All nuclei labelled with the antibody.

H. Control

#14/39P.14. No nuclei appear to be labelled.

I. Brn3.1 Antibody

#15/39P.14. All nuclei labelled with the antibody.

J. Control

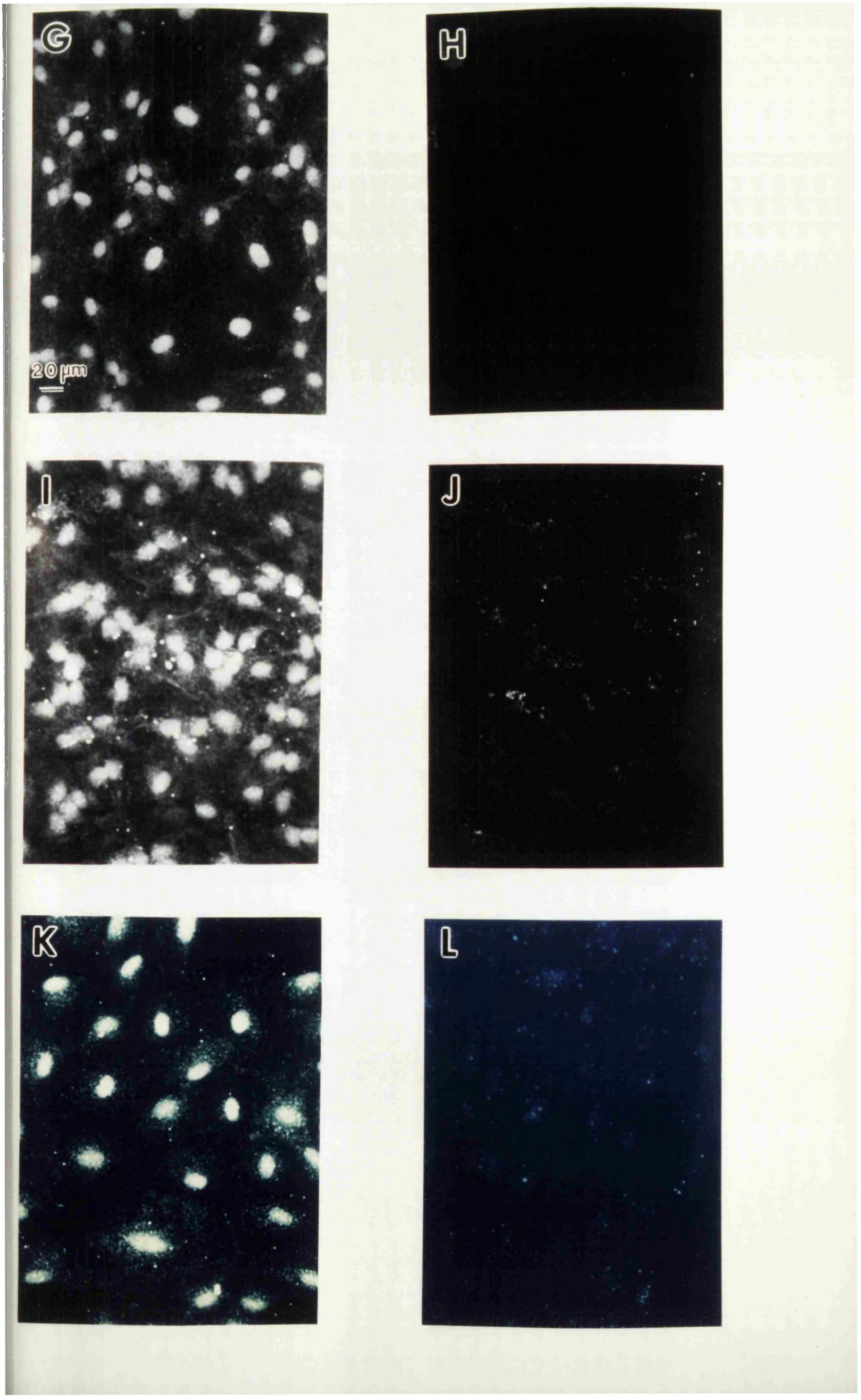
#15/39P.14. No nuclei appear to be labelled.

K. Brn3.1 Antibody

#24/39P.14. All nuclei labelled with the antibody.

L. Control

#24/39P.14. No nuclei appear to be labelled.


G
20 µm
H
I
J
K
L

3.3 Characterisation of clones #14, #15 and #24

The clones were characterised to determine the expression of epithelial cell markers and hair cell specific proteins and gene products under differentiating conditions. Cell cultures were grown for two weeks at 39°C before the requisite samples were collected.

3.3.1 Epithelial characteristics

Six random cell counts using a microscope counting grid were performed. Each count contained approximately 40 cells, for each clone immunolabelled at 33°C and 39°C. In the controls for vimentin, cadherin, and cytokeratin the primary antibody was omitted and normal serum of the same species as the antibody was added. There was no control for the actin immunolabelling. All controls were negative at 33°C and 39°C (data not shown).

At 33°C, in #14 an #15 all cells expressed vimentin and actin and none of the cells expressed cytokeratin or cadherin. In #24, at 33°C, all cells expressed vimentin and actin, 67% cells expressed cytokeratin and none expressed cadherin (Fig.3.5).

At 39°C, in #14, #15 and #24 vimentin was downregulated in all cells. Actin became more organised creating circumferential whorls and possibly microvilli. Likewise, in all clones cadherin labelling showed adherens junctions between cells and in #24, 63% of cells expressed cytokeratin. There was no statistically signficant difference in #24 between the percentage of cells labelled with cytokeratin at 33°C and 39°C. DAPI was used to double label the nuclei of the cells at 33°C and 39°C. A summary table (Fig.3.14) of the immunohistochemistry in #14, #15 and #24 is presented on page 69.

FIGURE.3.5

Vimentin, actin, pan-cadherin and pan-cytokeratin immunolabelling of #14, #15 and #24 at 33°C and 39°C. (x400).

Representative pictures have been taken to demonstrate the salient features of each antibody labelling.

33°C

A. Vimentin Antibody. B. Dapi labelling nuclei of same sample.

#24/33P.21. All cells labelled with antibody.

C. Texas-Red Phalloidin. D. Dapi labelling nuclei of same sample.

#24/33P.21. All cells labelled phalloidin

E. Pan-Cadherin Antibody. F. Dapi labelling nuclei of same sample.

#14/33P.23. Background labelling only. No organised pan-cadherin labelling was seen between the dividing cells.

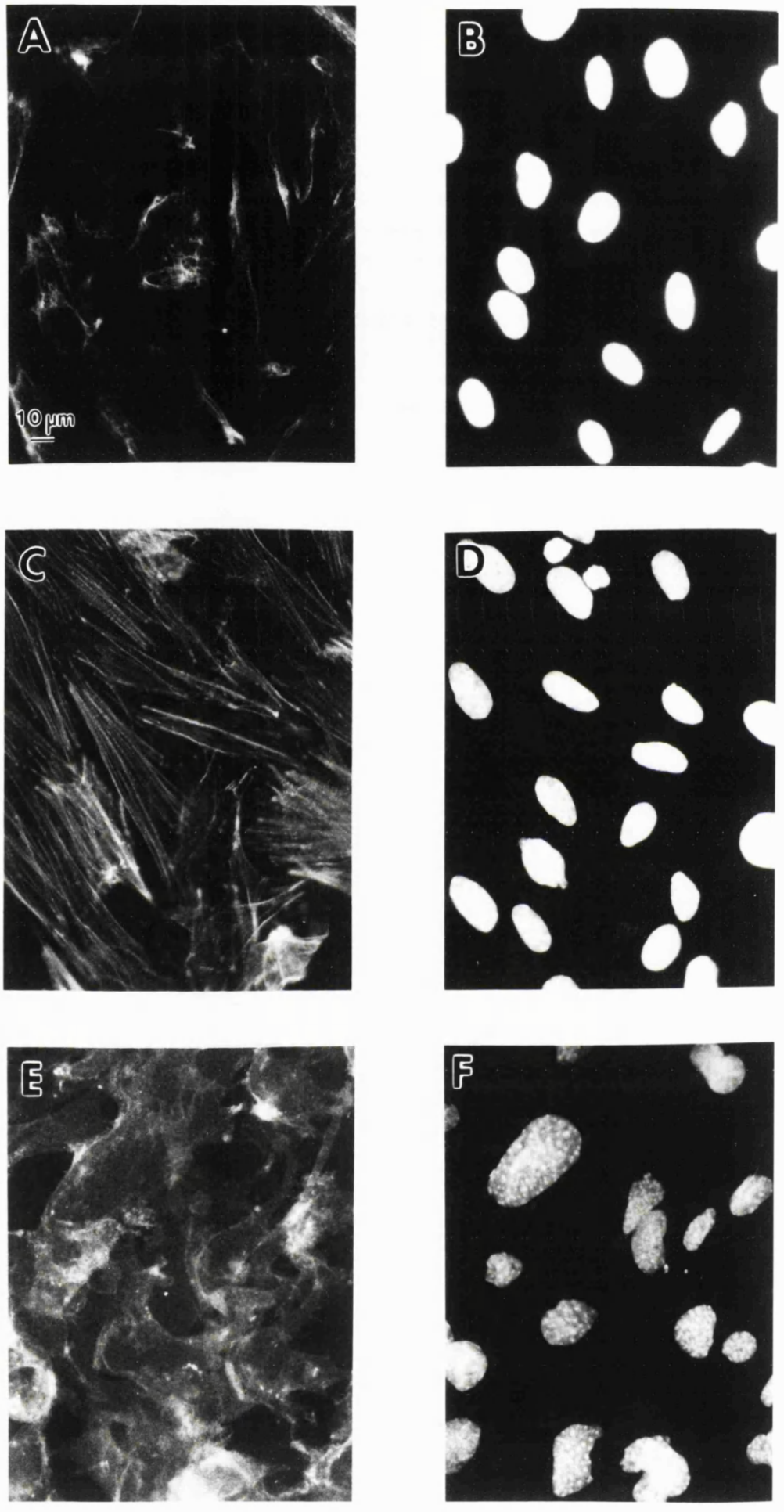

A
10 μm
B
C
D
E
F

FIGURE.3.5 continued.

39°C

G. Vimentin Antibody. H. Dapi labelling nuclei of same sample.

#15/39P.21. Downregulation of vimentin in all cells.

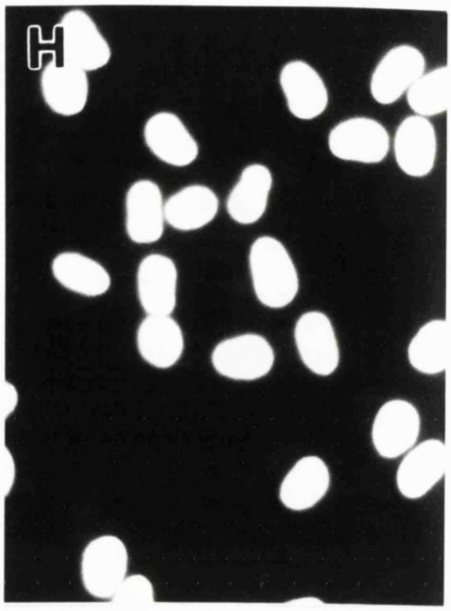
I. Texas-Red Phalloidin. J. Dapi nuclei of same sample.

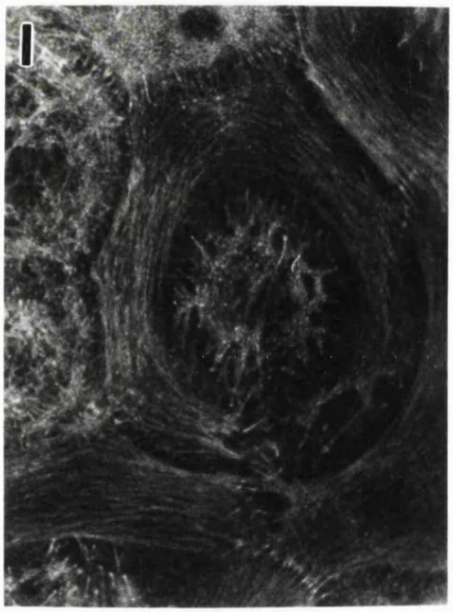
#14/39P.22. Actin becomes more organised creating circumferential whorls. Stress fibres can also be seen between cells.

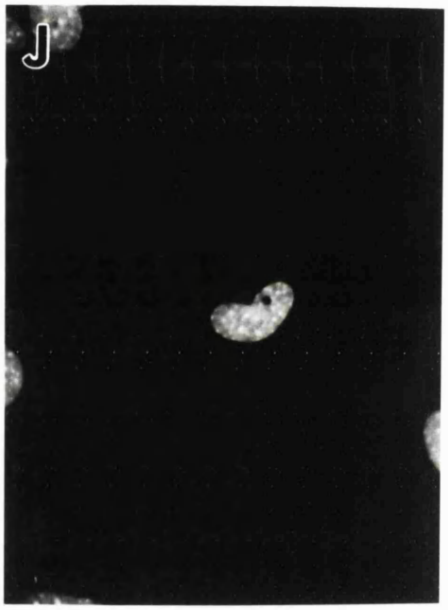
K. Pan-Cadherin Antibody. L. Dapi labelling nuclei of same sample.

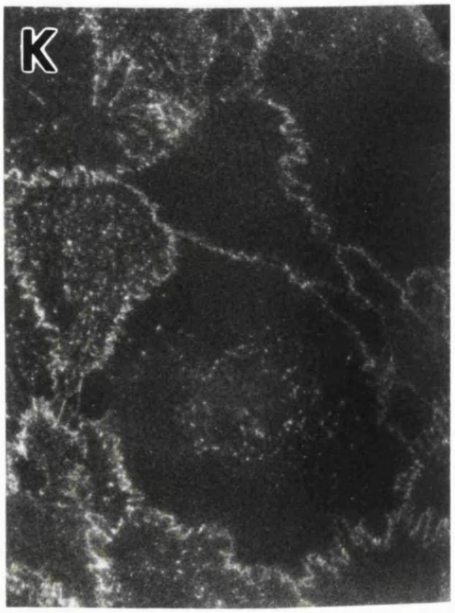
#14/39P.22. Pan-cadherin antibody defines clear polygonal boundaries around the cells' perimeter.


G
10 µm


H


I


J


K

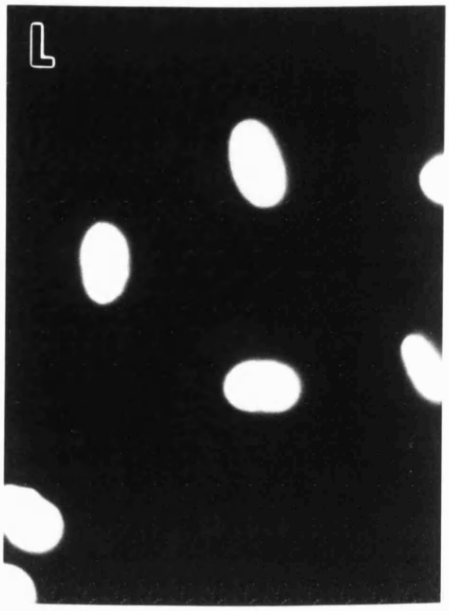

L

FIGURE.3.5 continued.

M. Pan-Cytokeratin Antibody. N. Dapi labelling nuclei of same sample.

#24/33P.21. Cytokeratins were expressed in the cells.

O. Pan-Cytokeratin Antibody. P. Dapi labelling nuclei of same sample.

#24/39P.20. Cyotokeratin expression shows a dense uniformly staining organised network of perinuclear filament bundles in the cells. The cytoarchitecture changes as the cells differentiate and the cyokeratin becomes more distributed throughout the cell. Arrows depict cells which have not been labelled with Pan Cytokeratin antibody.

R. Double labelling with Pan-Cytokeratin Antibody and Dapi at 39°C.

#24/39P.20. Double labelling demonstrates more clearly that some of the cells are not labelled with pan-cytokeratin antibody.

S. Double labelling with Pan-Cytokeratin Antibody and Dapi at 33°C. (x200).

Representative picture of #14/33 and #15/33 demonstrating that no cells are labelled with pan-cytokeratin antibody. Similar results were found at 39°C (data not shown).

M
10 μm

N

O

P

R

S
4
20 μm

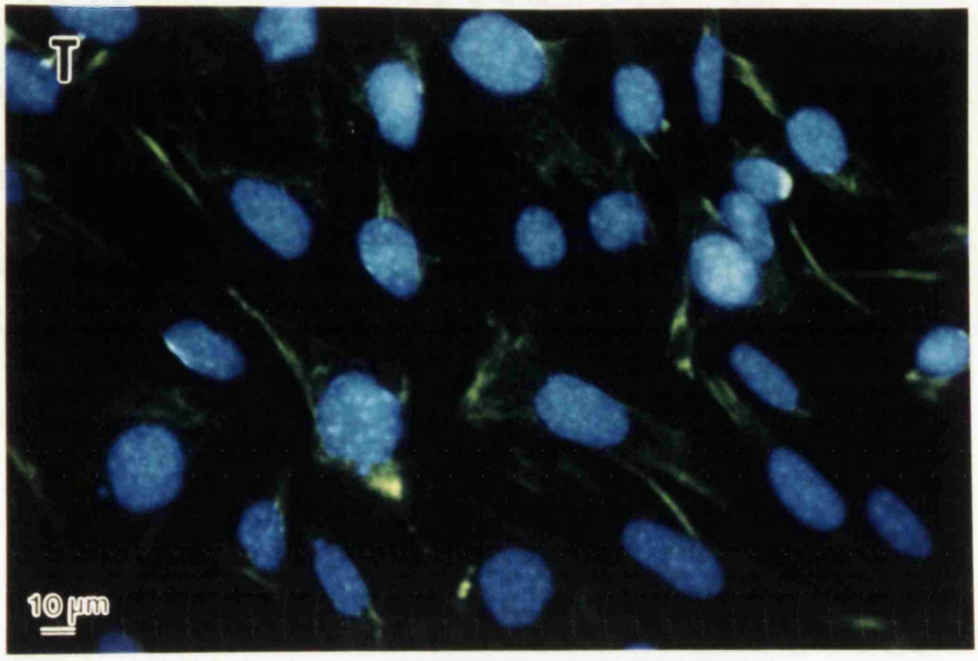
FIGURE.3.5 continued.

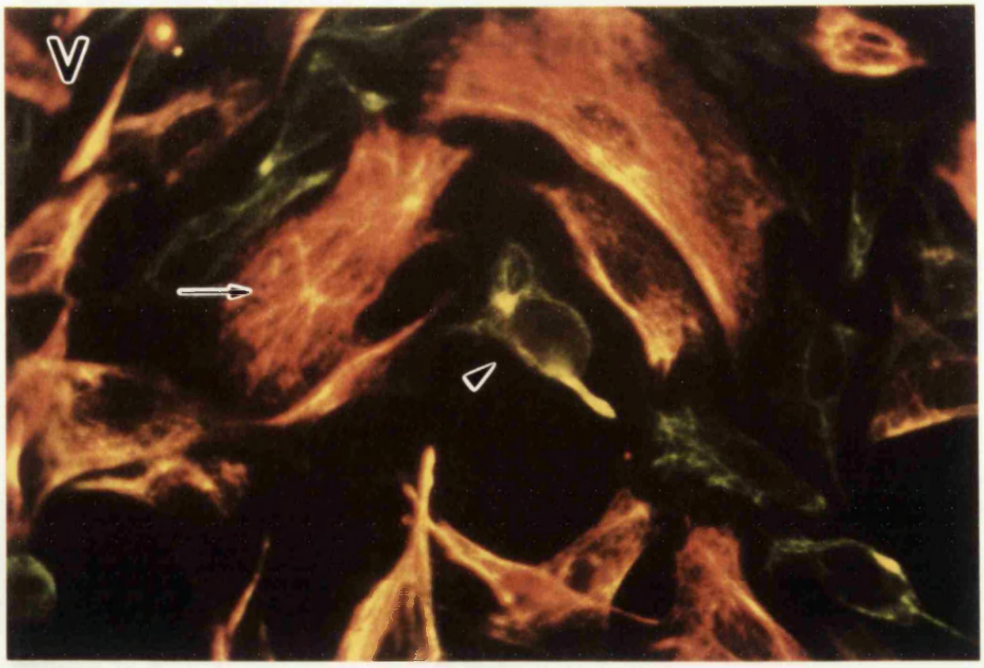
T. Double labelling with Vimentin Antibody and Dapi at 33°C. (x400).

#14/33P.23. Double labelling demonstrating that nearly all cells express vimentin.

V. Double labelling with Vimentin Antibody and Pan-Cytokeratin at 33°C. (x400).

#24/33P.21. Double labelling showing that some of the cells are only labelled with both antibodies in different proportions (arrows and arrowheads) whilst other cells are labelled with vimentin (green labelling).


T
10 µm


V

3.3.2 Hair cell characteristics

mRNA and protein extracts, collected at 33°C (#14P.20, #15P.19, #24P.19) and after 14 days at 39°C (#14P.19, #15P.18, #24P.18) for analysis by RT-PCR and western blot, were processed at the same time under the same conditions. For the western blots, previous experiments had shown that there was no cross reactivity between the secondary antibody and the protein extract.

The results were as follows (Fig.3.6). Analysis of the RT-PCR showed that at 33°C *Brn3.1* was present in #14, #15 and particularly in #24. At 39°C, there was similar expression of *Brn3.1* in #14 and #15 as at 33°C but it was downregulated in #24. *Myo7a,* at 33°C, was present in #14 and #24 but barely expressed in #15. At 39°C, it was expressed at similar levels in #14 and #15 as at 33°C and it was upregulated in #24. *α9* could not be detected by RT-PCR in #14, #15 and #24 at either 33°C or 39°C (results not shown).

Analysis of the western blots showed that at 33°C Brn3.1 was present in #14 and #24 and at slightly higher levels in #15. At 39°C, Brn3.1 was upregulated in all three clones (Fig, 3.7). MyosinVIIA, at 33°C, was present only in #24. At 39°C, there were low levels of expression in #14 and #24 (Fig.3.8). Fimbrin, at 33°C, was present in all three clones and similar levels of expression are seen at 39°C in all three clones (Fig.3.9).

FIGURE.3.6

RT-PCR of *Brn3.1, Myo7a, jagged2* and *gapdh* in clones #14, #15 and #24 at 33°C and 39°C.

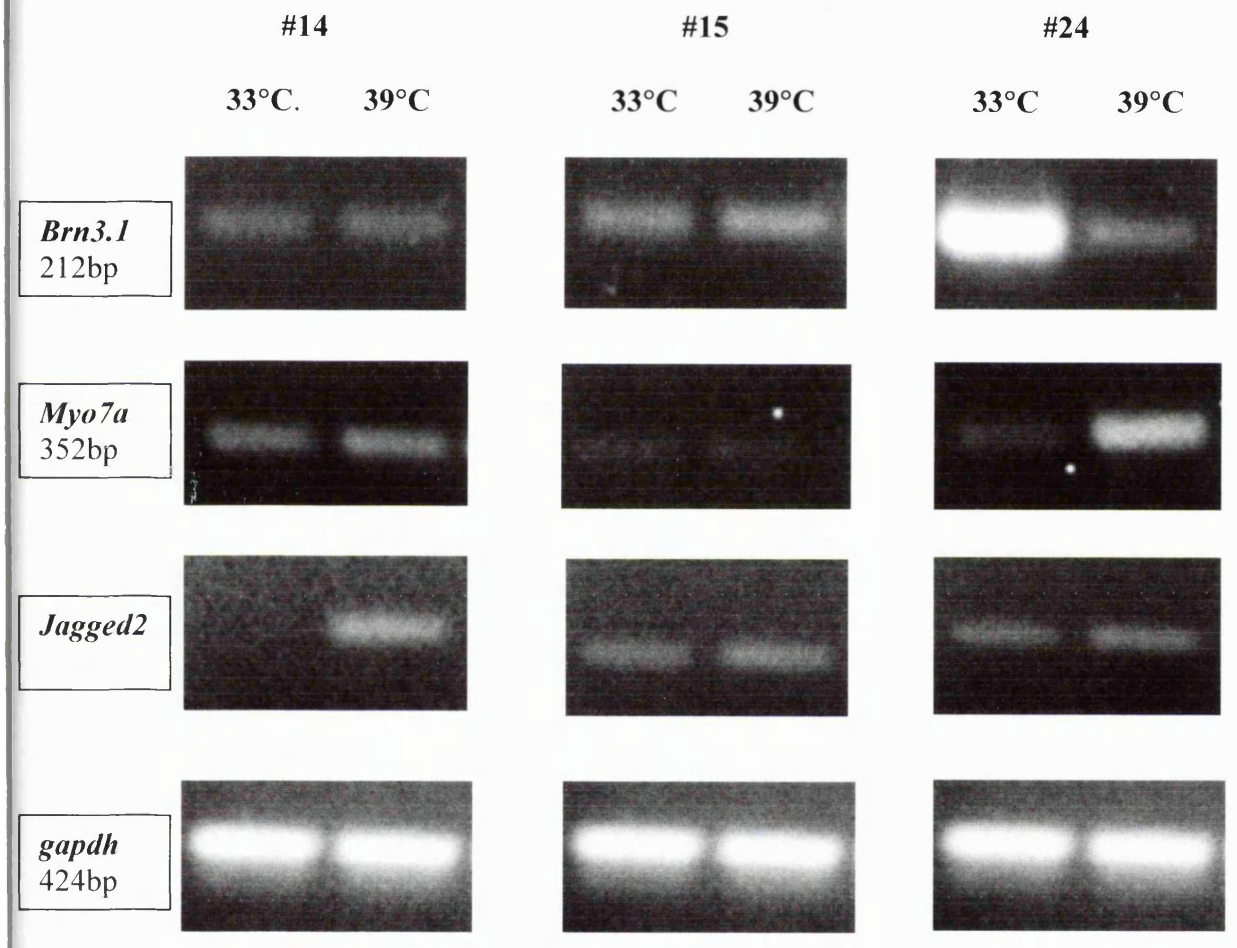


FIGURE.3.7

Western blot of Brn3.1 using a 14% SDS-Polyacrylamide separating gel in #14, #15, #24 and OC-1 (control) at 33°C and 39°C.

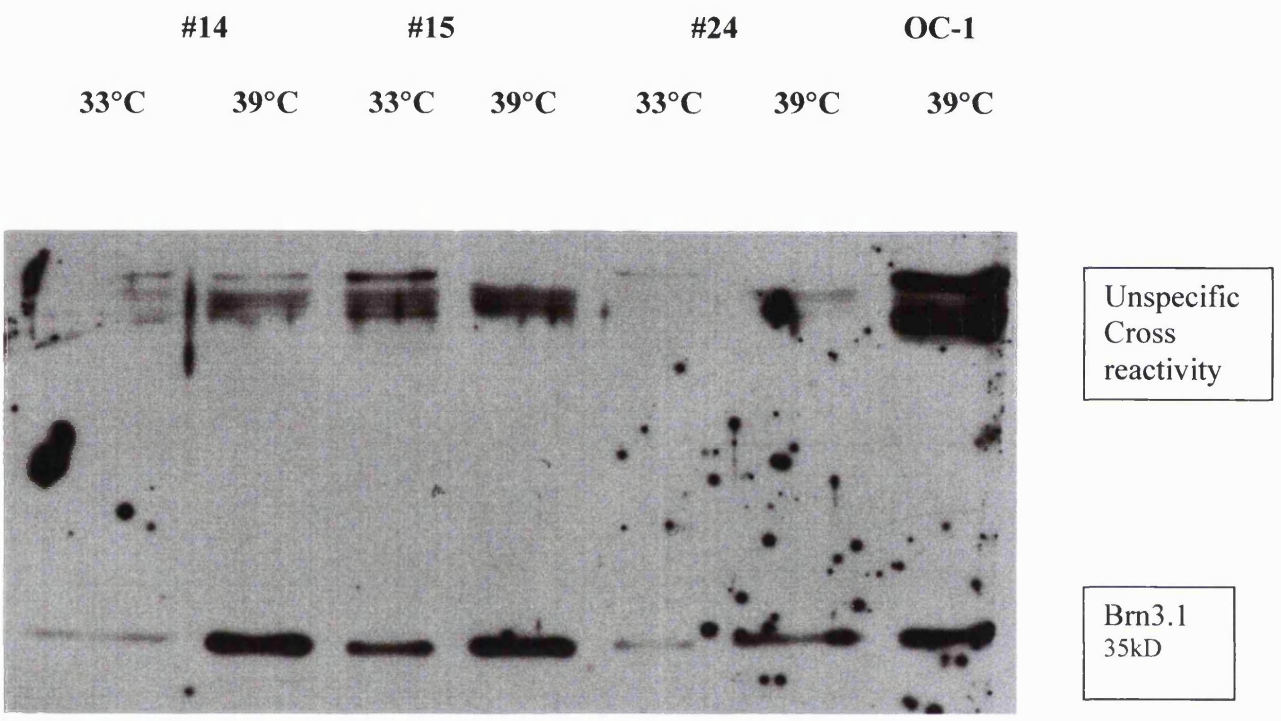


FIGURE.3.8

Western blot of MyosinVIIA using 7.5% SDS-Polyacrylamide separating gel in #14, #15, #24 and OC-2 (control) at 33°C and 39°C.

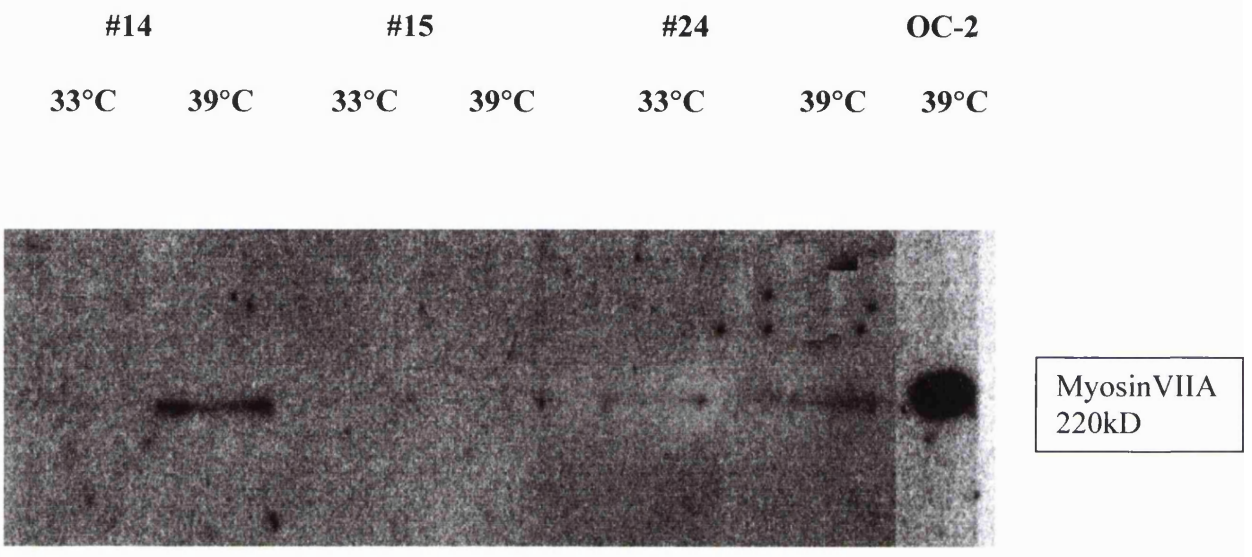
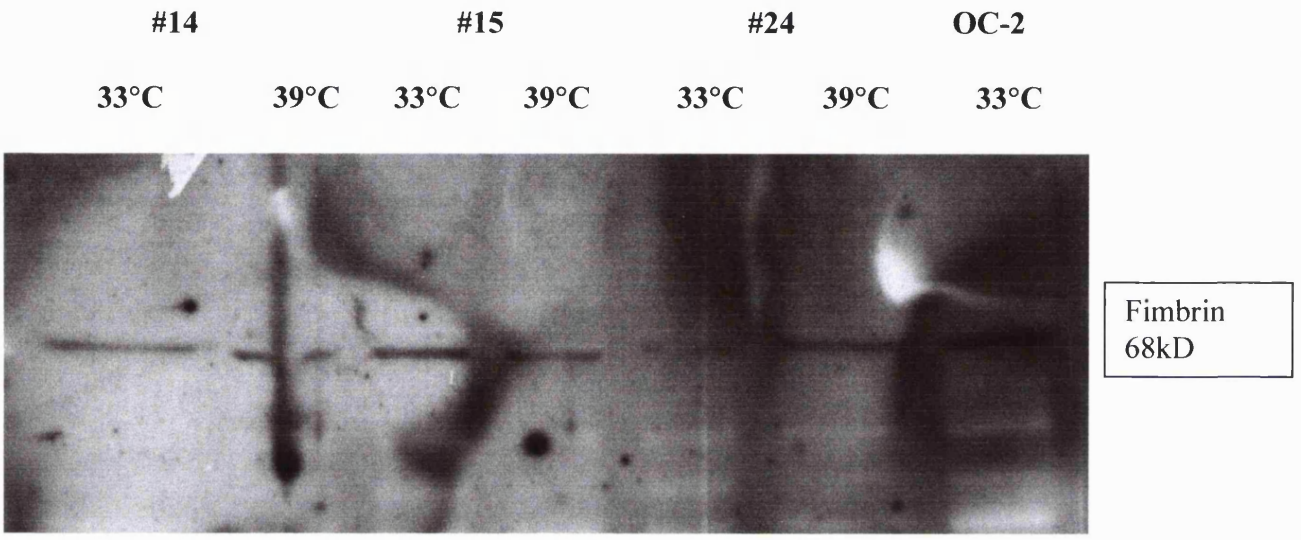


FIGURE.3.9

Western blot of fimbrin using 10% SDS-Polyacrylamide separating gel in #14, #15, #24 and OC-2 (control) at 33°C and 39°C.



3.4 Characterisation of the clones in relation to the mechanism of lateral specification

mRNA and protein was extracted from #14P.19, #15P.18 and #24P.18 at 33°C and from #14P.20, #15P.19 and #24P.19 at 39°C after 14 days to determine the expression of Notch1, Numblike, Jagged1, *jagged2* and OCP-2. As before for the western blots previous experiments had shown that there was no cross reactivity between the secondary antibody and the protein. *gapdh* and dH_2O were used as controls for the RT-PCR.

At 33°C, Notch1 and Jagged1 were expressed in #14, #15 and #24. At 39°C, there was no expression of Notch1 or Jagged1 in the clones (Figs.3.10 & 3.11). Numblike was expressed at equal levels in all clones at 33°C and 39°C (Fig.3.12). *jagged2* was present in #15 and #24 but not #14 at 33°C, whereas at 39°C it was upregulated in #14 and remained at similar levels to those at 33°C in both #15 and #24 (Fig.3.6). OCP-2 was not expressed in the clones at either 33°C or 39°C (Fig.3.13).

A summary table (Fig.3.14) of the results of the protein and gene expression in #14, #15 and #24 is presented on page 69.

FIGURE.3.10

Western blot of Notch1 using a 7.5% SDS-Polyacrylamide separating gel in #14, #15 and #24 at 33°C and 39°C.

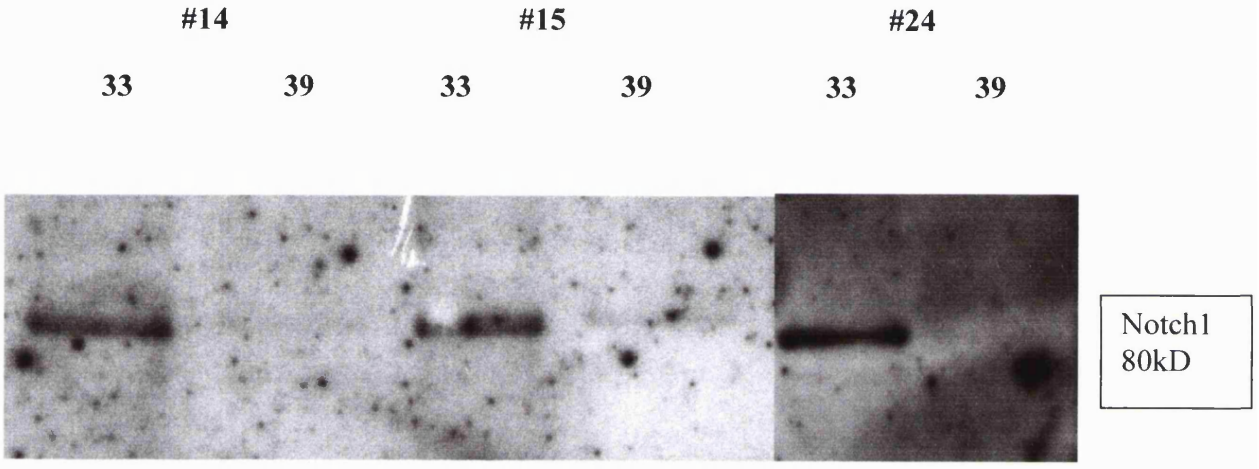


FIGURE.3.11

Western blot of Jagged1 using 7.5% SDS-Polyacrylamide separating gel in #14, #15 and #24 at 33°C and 39°C.

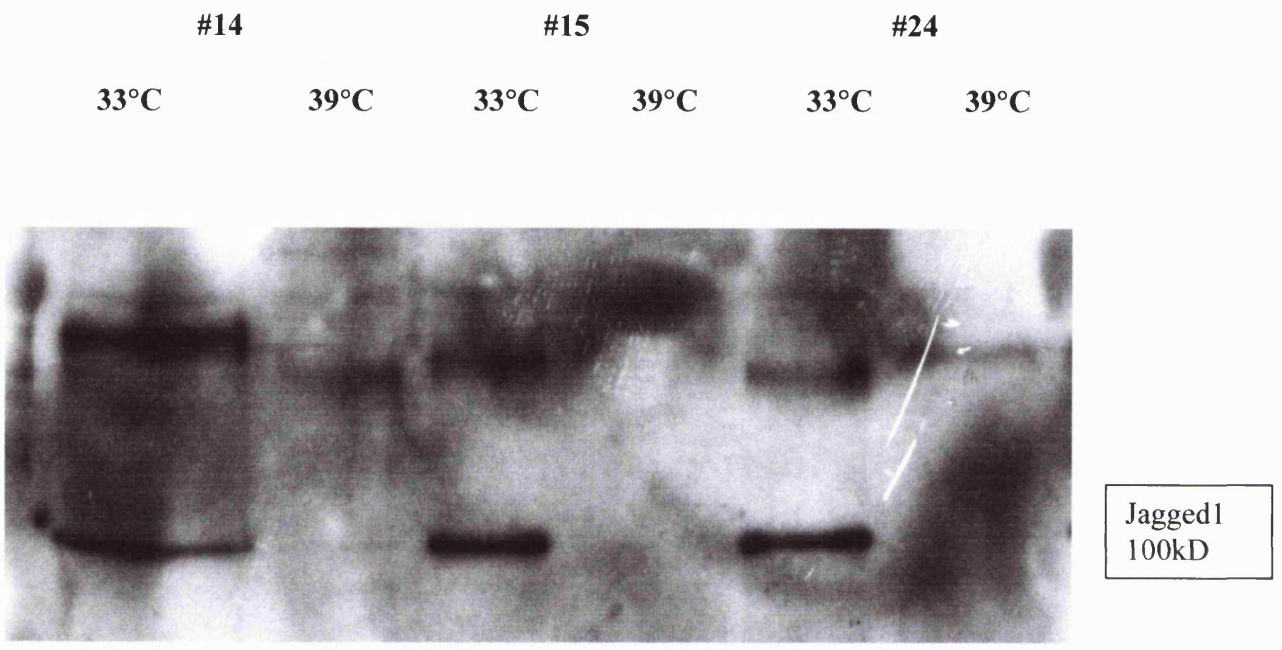


FIGURE.3.12

Western blot of Numblike using 10% SDS-Polyacrylamide separating gel in #14, #15 and #24 at 33°C and 39°C.

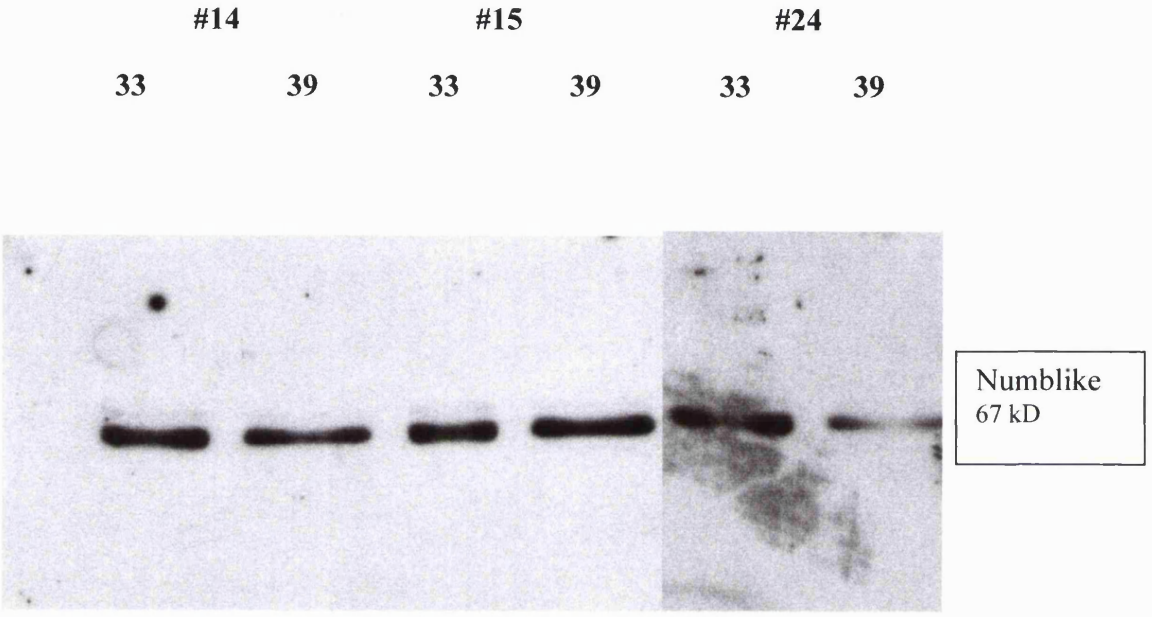
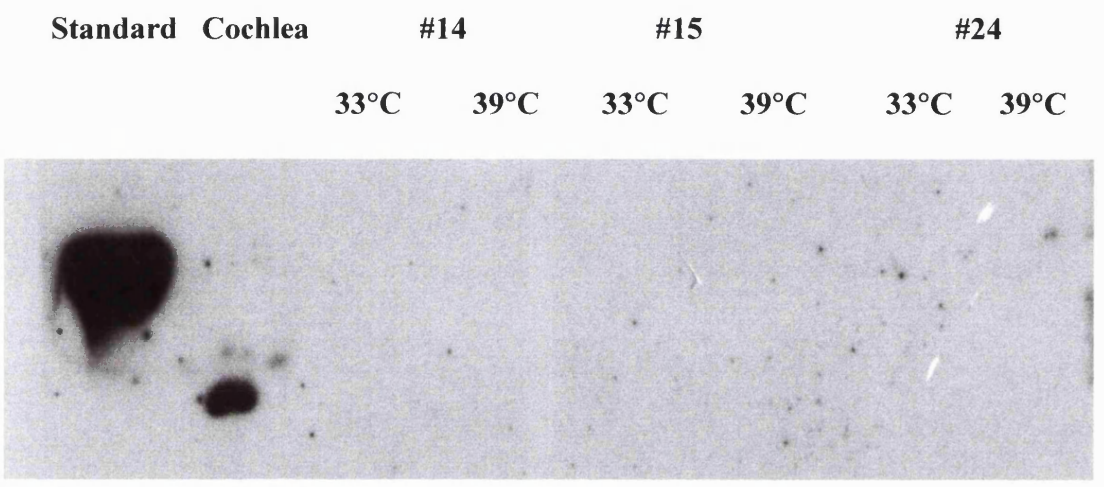


FIGURE.3.13

Western blot of OCP-2 using 14% SDS-Polyacrylamide separarting gel in #14, #15, #24 and cochlea extract (control) at 33°C and 39°C.



Product	#14/33	#14/39	#15/33	#15/39	#24/33	#24/39
Vimentin	+++	+	+++	+	+++	+
Cytokeratin	-	-	-	-	++	++
Cadherin	-	+++	-	+++	-	+++
Actin	+++ linear	+++ whorls	+++ linear	+++ whorls	+++ linear	+++ whorls
Brn3.1 PCR	+	+	+	+	+++	+
Brn3.1 WB	+	+++	++	+++	+	++
Myo7a PCR	+	+	+/-	+/-	+/-	++
Myo7a WB	+/-	++	+/-	+/-	+/-	+
Fimbrin WB	++	++	++	++	++	++
Notch1 WB	+++	-	+++	-	+++	-
Jagged1 WB	+++	-	+++	-	+++	-
Jagged2 PCR	-	++	++	++	++	++
Numblike WB	+++	+++	+++	+++	+++	++
OCP-2 WB	-	-	-	-	-	-

FIGURE.3.14

Summary table of results of the PCR's and WB's for the clones. (Comparisons can only be made between columns of a particular row).

CHAPTER FOUR

DISCUSSION

A differentiated cell can be characterised by the proteins it expresses. This study has demonstrated that *in vitro* cochlea hair cells do express some of the characteristic proteins that define a hair cell. Furthermore, the temporal expression of these genes *in vitro* is roughly equivalent to that recorded during normal development (Fig.4.1). This suggests that the *in vitro* system is running along similar developmental pathways and can therefore be used as a system for discerning specific points of interest about the development of auditory hair cells.

	33°C				39°C			
	#14	**#15**	**#24**	**In Vivo**	**#14**	**#15**	**#24**	**In Vivo**
Brn3.1	+	+	+	+	+	+	+	+
Myosin VIIA	+	-	+	+	+	-	+	+
Fimbrin	+	+	+	?	+	+	+	+
Notch1	+	+	+	+	-	-	-	-
Jagged1	+	+	+	+	-	-	-	-
Jagged2	-	+	+	-	+	+	+	+

FIGURE.4.1. *In vitro* and *in vivo* expression of the products analysed in this study at 33°C and 39°C (equivalent to approximately E14 and P6 respectively *in vivo*). ("+" = present; "-" = absent).

4.1 *Brn3.1* and *α9AChR* expression under differentiating conditions

There are estimated to be several thousand genes encoding transcription factors, and a substantial number of these are probably expressed in the inner ear. Doubtless it will be several years before a complete understanding of their role is known in the organogenesis of the cochlea. However, work on the transcription factor family Brn3 has demonstrated just how important they are in the development of the cochlea particularly the critical role *Brn3.1* plays in the differentiation of hair cells. Both auditory and vestibular hair cells require *Brn3.1* for differentiation (Ryan *et al.*, 1997) and it can be considered as a definitive hair cell marker. In the mouse, Xiang *et al.*, (1998) found Brn3.1 protein expression begins at E12.5 in scattered cells of the presumptive cochlear sensory epithelium.

In mice, auditory function begins at P.8 and becomes "adultlike" at P.14 (Alford and Ruben, 1963). Therefore, after 14 days in culture the cells would have nominally reached between P.7-P.10 *in vivo,* the time at which the cochlea begins to function. In UB/OC-1 (Fig.3.1a) *Brn3.1* was barely detectable at 33°C but expression was present by day 3, nominally E16, and continued to be expressed for 30 days under differentiating conditions. The difference between the *in vivo* and *in vitro* expression of *Brn3.1* could lie in the time taken for differentiation to occur along the organ of Corti from the base (E14) to the apex (E18) (Nishida *et al.*, 1998). UB/OC-1 could be derived from any region along the cochlea epithelium and the time taken for the TAg to be switched on when the sensory epithelium was first extracted must also be taken into account.

α9 mRNA expression was not detectable until after day 3 and continued to be expressed for a month. Three important inferences can be drawn from these results. Firstly, the expression of both *Brn3.1* and *α9* is conditional. Secondly, *Brn3.1*

expression occurs before that of *α9*. However, as *α9* was not expressed by the clones using RT-PCR it is possible that there is no causal link between *Brn3.1* and *α9*, or more likely that other factors other than *Brn3.1* are required for the regulation of *α9*. Thirdly, the expression of *α9* occurs between 3-6 days *in vitro,* nominally E17- E20 (Fig.3.1b), which corresponds to the *in vivo* expression of *α9* at about E18 in rat (Luo *et al.*, 1998). This provides some verification of the temporal validity of the *in vitro* system in comparison to the *in vivo* system. Brn 3.1 may control other genes. Recent work by Ryan *et al.*, (1998) has suggested that the pLIM homoedomain transcription factor maybe a downstream target of Brn3.1 and possibly controlled directly by it. Another possible downstream target is the promoter of the gene encoding the synaptic vesicle protein SNAP-25 (Smith *et al.*, 1998). Smith *et al.*, (1998) transfected neuronal cells with Brn3.1 and found that the SNAP-25 promoter was activated by the N-terminal activation domain of Brn3.1.

Although Brn3.1 is essential for hair cell development it is unlikely to be the commitment factor. Xiang *et al.*, (1998) demonstrated that in Brn3.1$^{-/-}$ mice, hair cells could be initially generated to undergo differentiation but they did not mature to grow stereociliary bundles and a small number of the cells failed to migrate into the hair cell lumenal layer. They proposed that Brn3.1 must play a crucial role in the maturation, migration and survival of hair cells but is not required for commitment and initial differentiation. This action of Brn3.1 is consistent with the action of other members of the Brn3 family (Brn3.0 and Brn3.2) which function in a similar mode during mammalian neural development. That is, they are required for differentiation, maintenance and/or migration of sensory neurons, but are not involved in fate commitment (Gan *et al.*, 1996; McEvilly *et al.*, 1996; Xiang *et al.*, 1996; Xiang *et al.*, 1998). Rather, there must be an upstream regulator of Brn3.1 which determines hair

cell fate. In order to explore this possibility the cell line UB/OC-1 was cloned in an attempt to create Brn3.1 negative cell lines.

4.2 Cloning UB/OC-1 to generate Brn3.1 negative cell lines.

From the results UB/OC-1 is not clonal and the sub-clones probably represent cells derived from different areas of the organ of Corti and therefore were immortalised at different stages of differentiation. Although the probability that the sub-clones are clonal is 99%, there is no absolute guarantee that the derived population is the progeny of one cell. This has always to be borne in mind when dealing with clonal cell lines. As clones #14 and #15 appeared negative for Brn3.1 immunolabelling at 33°C and positive at 39°C I propose that they represent an earlier stage of differentiation prior to the activation of *Brn3.1*. In contrast, #24 represents a later stage of differentiation as it is positive for Brn3.1 at both 33°C and 39°C. This difference is possible for the following reason. The cells were derived at a critical embryonic stage when Brn3.1 is initially expressed and when hair cell differentiation occurs temporally from the base to the apex with a delay of 2-3 days. The clones may represent different locations along this gradient.

Importantly, all the cells express Brn3.1 at 39°C suggesting that they are all hair cells and that they are already committed to a hair cell fate. A possible explanation for all the cells expressing Brn3.1 is that at the time when UB/OC-1 was undergoing selective adhesion the cells were separated from one another and there was therefore no induction of Notch1. Thus the cells naturally followed their primary fate which is to become hair cells.

4.3 Characterisation of clones #14, #15 and #24

Pan-cadherin shows specific reactivity against E, N, P, V, R and T cadherin. Pan cytokeratin is a monoclonal antibody, which recognises human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19. Cytokeratins 1, 4, 5, 6, and 8 are members of the type II (basic) subfamily. Cytokeratins 10, 13 and 18 are members of the type I (acidic) subfamily. Cytokeratin 19 is a type I keratin which is expressed in both simple and stratifying epithelia (Moll *et al.*, 1982a). The formation of cytokeratin filaments requires heteropolymerisation of cytokeratins from both the acidic and basic subfamilies. Monoclonal anti pan-cytokeratin antibodies are used as specific markers of epithelial cell differentiation and have been used widely as markers in tumour identification and classification.

The intermediate filaments tend to be expressed in a cell lineage specific fashion: for example, vimentin is expressed by mesenchymal cells, such as fibroblasts, whilst cytokeratins are expressed by epithelial cells. Unusually, within the inner ear vimentin is expressed by the sensory epithelium during development. In all the clones the expression of vimentin is downregulated during differentiation (Fig.3.5A and Fig3.5G) suggesting that the clonal cell lines have epithelial characteristics that change with development. Indeed, it has been suggested that the maximal expression of vimentin may precede a cell's commitment to terminal differentiation (Ngai *et al.*, 1984). The results of the vimentin expression are consistent with Kuijpers *et al.*, (1992) who demonstrated similar expression in the rat inner ear during development. The transient expression of vimentin in the epithelial lining of the developing inner ear concurs with comparable observations on developing epithelia at other sites of the body such as kidney tubules and the genital ducts (Viebahn *et al.*, 1987).

The presence of prominent cytoplasmic cytokeratin filaments is a distinguishing feature of the epithelial phenotype (Sun and Green, 1978b; Moll *et al.*, 1982). Furthermore, cytokeratins have been extensively studied and found to be particularly useful markers of normal and experimentally altered patterns of epithelial differentiation (Moll *et al.*, 1982). The presence of cytokeratin expression in #24 at 33°C and 39°C suggests that this cell line has retained this epithelial morphological characteristic. Although a similar percentage of cells in #24 express the protein under both conditions, the distribution of the protein has altered. At 39°C, the cell culture shows a uniformly organised network of perinuclear filaments consistent with differentiation. In the developing rat inner ear Kuijpers *et al.*, (1991b and 1992) found that cytokeratin was downregulated in hair cells during embryonic development and became undetectable by immunofluorescence by about P4. Both #14 and #15 fail to express cytokeratin at 33°C and 39°C *in vitro*. At 39°C this could be taken as consistent with the *in vivo* experiments but at 33°C the lack of expression suggests that either the in vitro cells are not behaving in the same manner as the in vivo system or that other mechanisms are at play. It has been reported that cytokeratin expression can be distorted in cells transfected with the SV40 virus (Steinberg and Defendi, 1985). They found that cells maintained in culture past the period of growth crisis, which normally occurs between the 10^{th} and 15^{th} passage, fail to express cytokeratins. They concurred that this modulation of the phenotype was due in part to the virus, which promoted an undifferentiated or more immature state. As clone 24 does express cytokeratin under both conditions it is possible that the lack of cytokeratin expression in #14 and #15 could be due to the effect of the TAg.

The co-expression of vimentin and cytokeratin has been widely reported in developing epithelia (Lane *et al.*, 1983; Viebahn *et al.*, 1988) and in epithelial cells

grown in culture (Virtanen *et al.*, 1981). In the developing inner ear, the co-expression of vimentin and cytokeratin has been reported in the epithelial lining by Wikstrom *et al.*, (1988) and Anniko *et al.*, (1987). Kuijpers *et al.*, (1992) also reported transient co-expression of vimentin and cytokeratin within the developing epithelial lining but not in the developing sensory epithelia. Only #24 at 33°C co-expressed vimentin and cytokeratin (Fig.3.5L) Although this is indicative of a differentiating cell it is not in agreement with *in vivo* data. Indeed little of significance can be concluded from the expression of vimentin or cytokeratin in the developing sensory epithelia *in vitro* in relation to the process of differentiation.

On the other hand the expression of cadherin and actin is more consistent with cells undergoing differentiation. Cadherin plays a critical role in cell adhesion and the assembly of individual cells into three-dimensional structures. Other than the work by Whitlon (1993), little has been published about the expression of cadherins in the developing organ of Corti. Whitlon showed that E-cadherin was first expressed at around E15 between the presumptive OHC's but not between the IHC's. In the P7 organ of Corti expression was limited to the supporting cells only. This downregulation of E-cadherin in the hair cells has also been noted by Forge *et al.*, (1997). However, this is not to say that all cadherins undergo a similar pattern of expression in the organ of Corti. At 33°C, all the clones label for pan-cadherins whilst at 39°C a regular polygonal pattern around the perimeter of the cells with interdigitation between cells is clearly seen (Fig.3.5K). This pattern of expression at 39°C in comparison to 33°C is suggestive of cells undergoing differentiation.

Actin is a protein that plays important roles in both muscle and non-muscle cells. It is one of the most abundant and highly conserved proteins in nature, comprising almost 10% of the total cellular proteins in some cells (Slepecky and Savage, 1994). In

mammals, at least six different isoforms are known (Vandekerckhove and Weber, 1978), of which four predominate in muscle. In mature hair cells β and γ-cytoplasmic isoforms are present (Slepecky and Savage, 1994) with γ-actin playing the predominant role in chick hair cells (Hofer *et al.*, 1997). The phalloidin toxin recognises both variants. In culture, under proliferating conditions actin is organised into antiparallel stress fibres (Fig.3.5C) between points of focal contact with the petri dish. At 39°C, there is reorganisation of the actin cytoskeleton. The actin now appears circumferentially around the perimeter of the cell with attachments to the lateral cell membrane, probably the zonula adherens (Fig.3.5I).

Taken together the expression of cadherin and actin at 39°C points towards a cell undergoing differentiation. However, with the knowledge from the previous experiments that all the clones express Brn3.1 at 39°C, a better marker of hair cell differentiation would be the conditional expression of specific hair cell proteins such as fimbrin and myosinVIIA.

The ontogenesis of stereocilia is essential for determining the shape and function of hair cells (Hudspeth and Jacobs, 1979). In hair cells fimbrin cross-links the β-cytoplasmic actin isoform in the stereocilia forming rigid actin bundles. In developing rat auditory hair cells Zine *et al.*, (1995) found fimbrin expressed at E.18 (a comparable mouse date would be E.16). There was an increase in the levels of expression with a concomitant temporal spatial relationship of expression from base to apex and from IHC to OHC. They concluded that the increase in fimbrin expression correlated with the maturation of the stereocilia. In all clones the expression of fimbrin remains constant at 33°C and 39°C (Fig.3.9). The finding that fimbrin is expressed at 33°C suggests that it may be an early hair cell marker.

In #15 there appeared to be virtually no expression of myosin VIIA from either the RT-PCR or western blot at 33°C or 39°C. This could reflect that either this clone is failing to differentiate properly, or that there is some intrinsic peculiarity of this cell line, which is exemplified by the lack of myosinVIIA expression. However, as both #14 (Fig.3.8) and #24 (Fig.3.6) upregulate the expression of myosinVIIA then some degree of differentiation has occurred. Importantly, the presence of the message at 33°C in #14 and #24 is consistent with the expression of message found *in vivo* by Sahly *et al.*, (1997) and Xiang *et al.*, (1998). As the expression of fimbrin and *myo7a* occurs, like *Brn3.1*, at 33°C it is possible that the differentiation of a hair cell may follow a number of parallel pathways before full development occurs, rather than follow a serial sequence of events where fimbrin and *myo7a* are expressed after *Brn3.1*. In addition, as the expression of *Brn3.1* decreases in #24 at 39°C whilst the expression of *myo7a* increases in #24 at 39°C this could suggest that *myo7a* is not under the direct control of *Brn3.1*. It may be that there is a gene or genes that control the initial differentiation of a hair cell and that fimbrin, *myo7a* and *Brn3.1* are the downstream targets of this gene/genes. They then follow their own pathways in parallel such that the end product is a fully differentiated hair cell. The question also remains as to why, despite the expression of these specific stereocilia proteins, there are no observed stereocilia in the cells at 39°C.

Although *α9* mRNA was expressed in UB/OC-1 and the receptor was found to be functionally active (Rivolta *et al.*, 1998), the lack of *α9* mRNA expression in the clones at 39°C reveals an important issue. That is, individually the cells within each clone either do not receive the necessary signals or factors from the neighbouring cells within the clone to produce the α9 receptor or that the level of *α9* mRNA was too low to be detected. Interestingly, when #14 and #15 were mixed together, *α9*

mRNA was expressed at 39°C (data not shown). In each case the culture conditions remained the same so the fact that the clones do not express *α9* and then do again when mixed would suggest that there is some dynamic interaction between them.

The expression of *Brn3.1* was re-examined in the clones to validate the previous experiment in UB/OC-1. Unexpectedly #14 and #15 expressed both the message and the protein at 33°C indicating that these are not *Brn3.1* negative cell lines as previously thought. Rather the antibody labelling was probably not sensitive enough to detect low levels of Brn3.1 at 33°C. There is conflicting evidence between the RT-PCR and western blot in #14 and #15 at 39°C. The former shows no conditional expression of *Brn3.1* whereas the latter does. This suggests that the level of message has remained the same by day 14 whilst the level of protein expression increases.

By contrast, in #24 the level of message is downregulated whilst the level of protein expression is also upregulated. This contradictory pattern of expression of the message (mRNA) could indicate that at the time the original cell line, UB/OC-1, was immortalised some cells had already begun to express high levels of Brn3.1 message (#24) i.e. they were already undergoing the process of differentiation. As the cells were becoming more differentiated less message was needed though the protein was still required. Arguably #24 could have been derived from the base of the cochlea where differentiation is known to begin first (Nishida *et al.*, 1998). Likewise #14 and #15 could be derived from a more apical region as they express relatively lower amounts of *Brn3.1* at 33°C.

Despite the fact that #14 and #15 were no longer *Brn3.1* negative at 33°C and therefore had passed that period of fate determination it was still important to elucidate the mechanism of lateral specification as relevant to the *in vitro* system.

4.4 Characterisation of the clones in relation to the mechanism of lateral specification

In amphibia and birds hair cells and supporting cells arise from a common precursor (Jones and Corwin, 1996; Fekete *et al.*, 1998). The results from this study demonstrate that some of the proteins and genes necessary for the mechanism of lateral specification are present in the clones. Importantly, their pattern of expression is similar to that reported elsewhere in the mouse and chick (Lanford *et al.*, 1998; Adam *et al.*, 1998). Indeed, the evolutionary conservation of this mechanism through the species from the sensory bristles of insects to the fine grained topography of the mammalian organ of Corti has been well described (Artavanis-Tsakonas *et al.*, 1995; Adam *et al.*, 1998; Haddon *et al.*, 1998).

#14, #15 and #24 express the proteins, Numblike, Notch1 and Jagged1 at 33°C. This is the first reported expression of the protein Numblike in cells derived from the embryonic cochlear epithelium. The Notch1 western blot (Fig.3.10) depicts bands of 80kD which are compatible with the expected weight of the activated proteolytic fragments of Notch1 (Notch intracellular domain [NICD]) as described by Schroeter *et al.*, (1998), Struhl and Adachi, (1998). However, the possibility that these bands are a proteolytic artefact caused by the manipulation of the sample cannot be excluded. The Jagged1 western blot (Fig.3.11) depicts bands of 100kD, less than the expected weight of 150kD. Other bands are present at the correct molecular weight at 33°C and it is likely that there has been proteolysis during the handling of the protein extract resulting in fragments of 100kD. The antibody was raised against the intracellular carboxy terminus of the ligand so the proteolysis is most likely to have occurred in the extracellular portion of the ligand.

The results suggest that these proteins are required early in the process of lateral specification by nascent hair cells. No precise point of time can be attributed to the expression of the proteins from the experimental results but *in vivo* studies have shown that the expression of *notch1* and *jagged1* begins at E13 (Lanford *et al.*, 1998). At 39°C, the expression of Notch1 and Jagged1 is downregulated whilst that of Numblike is maintained. Studies on the chick basilar papilla have shown a similar downregulation of expression of serrate1 (close homology to Jagged1) and Notch1 (Adam *et al.*, 1998).

jagged2 is upregulated in #14 and remains constant in #15 and #24 at both temperatures. *jagged2* is known to be expressed only in hair cells at E15 *in vivo* (Lanford *et al.*, 1998). The experimental results suggest that this pattern of expression is being maintained *in vitro*. All three clones continue to express *jagged2* at 39°C as expected in a hair cell population. The upregulation in #14 points towards this cell line undergoing differentiation later than #15 and #24 and is consistent with the previous data on *Brn3.1* expression.

In terms of understanding the mechanism of lateral specification a model is proposed that encompasses these results and results from the literature. From a group of equivalent cells in the prosensory cluster two distinct phenotypes emerge; the hair cell and the supporting cell. In a fashion similar to that in which a proneural cell gives rise to a neuron, shaft cell, socket cell and sheath to form a sensory bristle on a fly (Hartenstein and Posakony, 1990), so the hair cell and supporting cell are generated as a result of lateral specification. Whilst the cells in the prosensory cluster are undergoing mitosis just prior to their terminal mitosis the protein Numblike is symmetrically distributed amongst the progeny. At the point of terminal mitosis, *notch1* and one of its ligands *jagged1* are probably switched on by one of the

prosensory genes such as *Math1* (Shailam *et al.*, 1999). Thus two equivalent cells express Numblike, Notch1 and possibly Jagged1 or another of the many ligands such as Delta. In the chick basilar papilla and the zebrafish inner ear the ligand Delta appears transiently during the initial stages of hair cell fate determination (Adam *et al.*, 1998; Haddon *et al.*, 1998). In the mouse, the expression of *Delta1* in the inner ear appears to be limited to the nascent hair cells as well as some non-sensory areas (Morrison *et al.*, 1998). The next stage is unclear, but the hypothesis is that in one of the two cells (Cell B) Numblike is downregulated. In the other cell (Cell A) Numblike continues to be expressed (Fig.4.2). It then binds to Notch1 within the same cell (Guo *et al.*, 1996) and possibly blocks it. This sets up a bias in the signalling potential of the Numblike-bearing cell. This bias may be amplified by feedback regulation with the subsequent upregulation of the ligand in Cell A. The ligand activates the Notch1 in Cell B, which then activates *Hes1/5* via *RBP-J* with the subsequent downregulation of the prosensory genes. This in turn has the added affect via the regulatory feedback loop of upregulating Notch1 and downregulating the ligand in Cell B (Dorsky *et al.*, 1997). The downregulation of the ligand in Cell B results in the added downregulation of Notch1 in Cell A. As the prosensory genes are not deactivated in Cell A they continue with their function, which may well be the activation of differentiation with the concomitant expression of *Brn3.1*. Thus in this way the fate of the cell has already been determined before *Brn3.1* is switched on. Cell A follows the primary fate and becomes a hair cell and Cell B follows a secondary fate and becomes a supporting cell.

However, for some reason as yet unexplained Jagged1 expression appears to be downregulated in the nascent hair cells as development progresses and is replaced by another ligand Jagged2. It may well be that two ligands are required for cell fate

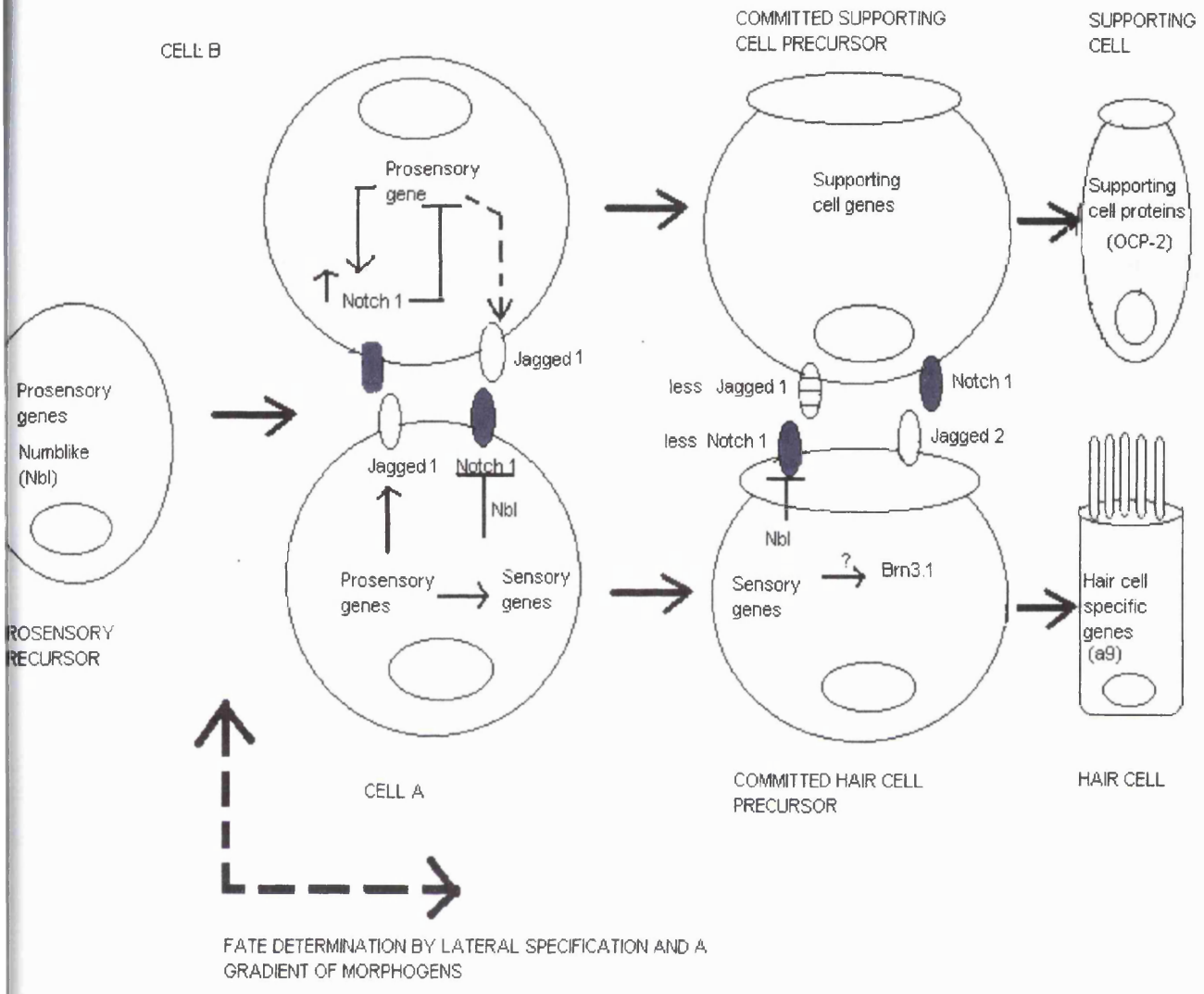


FIGURE.4.2 Model proposed for how fate determination occurs to generate a population of cochlea hair cells and supporting cells from a common precursor.

determination. The first is necessary to make the choice. The second is required to ensure that the cell fate is maintained until the cell has reached full maturity. Kimble and Simpson (1997) argue that duplicated ligands and receptors may have evolved to achieve the distinct spatial and temporal regulation of individual components. Alternatively, work by Zeng *et al.*, (1998) has shown the possibility of multiple Notch ligands serving redundant functions. Their results demonstrated in both sensory organ

cell fate specification and wing vein patterning that the function of one ligand is only apparent after the other ligand is removed. There are still many questions unanswered and the exact mechanism of how one set of cells become hair cells and another set become supporting cells has yet to be fully determined. Into this simplified hypothesis there are other factors to consider. These include, the role of the *fringe* genes and their modulation of the action of the ligand (Morsli *et al.*, 1998), and the role of the *Wnt* pathway and its interaction with Notch (Couso and Martinez Arias, 1994; Cadigan and Nusse, 1997).

4.5 Critical analysis of the use of cell lines in determining cochlear hair cell differentiation

This work has demonstrated that the cell lines are capable of establishing some important data in relation to the differentiation of cochlear hair cells. Firstly, the cell lines express the genes and proteins not only found in the mature hair cell but also those required for cell fate determination and differentiation. Secondly, the results have demonstrated that the *in vitro* system does follow reasonably well the temporal course of events *in vivo*. This is crucial if any inferences are to be made between the two systems and provides an opportunity for further experimentation into genes involved in differentiation, especially those under the control of key regulatory genes such as *Brn3.1*. From this work further research is currently being conducted into effects of *Notch1* and *Hes5* over-expression in UB/OC-1.

However, there are limitations to the immortal cell lines. Although they do provide a flexible and manageable system and can provide answers when looking at specific molecules at specific time points, they are not a means for completely unravelling the process of cell fate determination and differentiation in hair cells. Firstly, it is not

possible to make direct quantitative comparisons between *in vitro* and *in vivo* systems. For example; the levels of the intermediate filament protein glial fibrillary acidic protein (GFAP), in immortalised astrocytes are 10-20% of that aged-matched normal controls (Goodman *et al.*, 1993; Frisa *et al.*, 1994).

Secondly, the most striking observation are the cell's complete lack of any resemblance to a hair cell. This is in part due to the fact that the cells are grown on a two dimensional surface. But more importantly it could be due to the effect of the TAg on E-cadherin. E-cadherin is essential for the maintenance and function of epithelial cell layers and also plays a pivotal role in the very early development of an epithelium by defining the polarity of the cell (Larue *et al.*, 1994). Work by Martel *et al.*, (1997) showed that retinoblastoma inactivation by the SV40 TAg specifically induces a loss of epithelial cell markers including E-cadherin and cytokeratin in differentiated cultured epithelial cells. Lawlor (personal communication) has shown that whilst the Immortomouse cochlea explants do express E-cadherin, UB/OC-1 does not express it at 33°C or 39°C. Thus without the E-cadherin the cells are unable to create appropriate adherens junctions. Furthermore, the tight junctions are then unable to fully form without the presence of the necessary components of the adherens junctions. Apical and basal polarity is lost and consequently the defining characteristic of a hair cell: its stereocilia, is not constructed in the correct fashion. Although at least some of the necessary components of the stereocilia, myosinVIIA, actin, fimbrin are all present in the cells, the signals required for their correct morphological relationship are absent. Further experiments to overcome this problem include the transfection of E-cadherin into the cell line.

But the arguments are more complex, and intrinsic to the action of the SV40 TAg is its effect on retinoblastoma protein. Recently, Weinberg (1998) has commentated that

immortalisation with SV40 TAg merely represents a "pyrrhic victory" because the immortalising oncogene interferes with the differentiation of the cell. Differentiation appears to be associated with a particular domain of the pRb that is distinct from the domains that control proliferation (Sellers *et al.*, 1998). Thus the differentiation program of a cell is dependent on pRb. Weinberg continues by suggesting that perhaps the ectopic expression of telomerase, the enzyme that repairs the cells telomeres and thereby allows the cell to continue dividing, would be a better agent as it does not affect such regulatory systems as differentiation. However, the work of Kiyono *et al.*, (1998) has shown that despite insertion of the telomerase gene into cultured epithelial cells, the retinoblastoma (Rb)/$p16^{INK4a}$ tumour-suppresser pathway still needs to be inactivated in order for immortalisation to occur. Indeed it could be that the oncogene has the dual action of not only deregulating the advance of the cell cycle, but also inducing the expression of telomerase and preventing senescence.

Either way, provided the limitations of the SV40 TAg are understood it still remains a potent and reliable mechanism for immortalisation. In such systems as the cochlea it gives the opportunity for studying specific genes and their function in an otherwise difficult environment. To date the SV40 TAg has not been bettered as an immortalising agent in the inner ear.

4.6 Conclusion

Cell fate determination and differentiation depends on both cell-cell interactions and the effects of exogenous agents. This study has exclusively looked at cell-cell interactions and although aware of the effects that the culture medium may play upon the development of hair cells has not set out to identify these factors present in the serum. With this in mind the results reported have thrown light upon the mechanisms

that might underlie the fate determination of hair cells. It has also raised the issue of whether the differentiation of hair cells is a parallel event with many different strands running simultaneously. It has also reaffirmed that Brn3.1 is not required for hair cell commitment. Finally, it has shown that the *in vitro* model of conditionally immortalised cell lines is a viable means of examining specific events in the development of the cochlear hair cells.

Research on the development of the cochlea is in its infancy in terms of functional molecular biology but performing this will only be possible when a comprehensive descriptive analysis of cochlea development has been provided. This work has aided the descriptive analysis of cochlear hair cell fate determination and differentiation and looks forward to the exciting functional studies that lie ahead and the concomitant introduction of therapeutic strategies to manage sensorineural hearing loss, which afflicts so many.

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