# *IN VITRO* LONG-TERM DEVELOPMENT OF CULTURED INNER EAR STEM CELLS OF THE NEWBORN RAT

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*Running Title: Inner ear stem cells in vitro development*

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## ABSTRACT

The adult mammalian auditory receptor lacks any ability to repair and/or regenerate after an injury. However, the late developing cochlea still contains some stem cell-like elements that could be used to regenerate the damaged neurons and/or the organ of Corti's cells. Before using them in any application, the stem cell numbers need to be amplified because they are usually rare elements in late developing and adult tissues. The numerous re-explant cultures needed for the progressive amplification process can result in a spontaneous differentiation process. This fact has been implicated in the tumorigenicity of the stem cells when transplanted to a tissue. The aim of this study was to determine if cochlear stem cells could proliferate and differentiate spontaneously in long-term cultures without the addition of any factor that could influence these processes. Cochlear stem cells, which express nestin protein, were cultured in monolayers and fed with DMEM containing FBS (5%). Cultured cochlear stem cells quickly organized as typical spheres exhibiting a high proliferation rate, self-renewal property and differentiation ability. Secondary cultures of these stem cell spheres spontaneously differentiated into neuroectodermal-like cells. The expression of nestin, GFAP, vimentin and neurofilaments was evaluated to identify early differentiation. Nestin expression appeared in primary and secondary cultures. Other markers were also identified in differentiating cells. Further research may demonstrate spontaneous differentiation of cochlear stem cells and the teratogenic probability of these cells when used for transplantation.

**Keywords**: inner ear stem cells, deafness, cell culture, spontaneous differentiation.

## INTRODUCTION

Endogenous stem cells might enable the regeneration of adult tissue due to their proliferation and differentiation abilities (Martinez-Monedero and Edge 2007). However, sensory cells and neurons of the mammalian organ of Corti lack any regeneration ability because they are post-mitotic cells and cannot be replaced following an injury (Matsui et al. 2005; Lou et al. 2007; Oshima et al. 2007). Currently, the only means of improving hearing in sensorineural hearing loss is by cochlear implantation of an electrode array (Basura et al. 2009). These electronic devices enable a significant improvement in hearing function and speech. However, evidence of timbre differences and other perception aspects of auditory function (music audition, etc.), are not completely solved by cochlear implants (Limb 2006). Thus, improving the whole range of hearing should involve the replacement of damaged or degenerated sensory or neural cells from a source of progenitors (Hu and Ulfendahl 2006; Savary et al. 2007).

Two main auditory stem cell sources have been described: the utricular macula and the organ of Corti (Breuskin et al. 2008). In fact, the vestibular receptor still exhibits self-regeneration into adulthood. When vestibular receptor cells were cultured, they formed floating cellular spheres that expressed nestin (Li et al. 2003), a typical stem cells marker (Lendahl et al. 1990). Nestin-positive cells were identified in both developing and mature auditory receptor cells suggesting that they might be the precursors of sensory, supporting or neural cells (Malgrange et al. 2002; Kojima et al. 2004; Lopez et al. 2004). Clonal cell spheres were obtained from auditory receptor cultures of newborn rats supplemented with EGF and FGF-2. Cells obtained from

these spheres also expressed nestin and retained the ability to differentiate into supporting cells or cells resembling hair cells (Malgrange et al. 2002).

As previously described, the differentiation patterns of cochlear stem cells can be selected or influenced by specific factors added to the culture medium which determine the fate of the differentiating stem cell. The teratogenicity of stem cells in transplant assays is also well established. In fact, the culture conditions can determine tumorigenic capabilities of the stem cells. The teratogenicity of stem cells can be related to the proliferation rate, spontaneous differentiation rate and colony forming potential (Knoepfler, 2009). Therefore, the study of these characteristics in a specific type of stem cells may indicate the malignant potential of that particular cell in future transplant programs. The aim of this work was to determine whether cochlear stem cells from newborn rats might exhibit, in standard culture (with the addition of foetal bovine serum (FBS) only), signs of spontaneous differentiation. These spontaneous differentiation events can be relevant in future application of these cells in transplants to repair/regenerate the mammalian organ of Corti.

## MATERIAL AND METHODS

*a) Animals:*

A total of fifteen Wistar rat pups (P0) from five different litters were used for this study. Animals were housed in Central Animal House (Faculty of Medicine, University Complutense of Madrid, Spain). During this study, animal care and use was in strict accordance with the animal welfare guidelines of the Helsinki Declaration.

*b) Cell culture procedure:*

Dissociated cell cultures were obtained in aseptic conditions from auditory receptors of newborn (P0) Wistar rats using a simple method. Briefly, auditory receptors were removed from developing cochleae in Hank's buffered salt solution (HBSS; Sigma) (37ºC, pH 7.4). Pieces were gently mechanically dissociated with a sterile Pasteur pipette and placed in a 1mg/ml trypsin (Gibco) in HBSS solution, (15 mins, 37ºC). The cell suspension obtained was centrifuged (500 rpm, 4ºC, 5 mins) in a refrigerated centrifuge (Beckman GS-15R). The pellet was suspended in D-MEM (Gibco, Invitrogen, ref 41966-029) culture medium supplemented with 10% FBS (Biochrom AG).

The cell suspension was placed in a multiwell culture plate (Nunc; 12 wells) without any coating substrate. Cell cultures were incubated in standard conditions (37ºC and 5% $CO_2$ in humidified atmosphere) in a $CO_2$ incubator (Heraeus BB16). The medium was replaced every 2 days with fresh medium. Cultured cells were

studied and photographed using an inverted photomicroscope (Leica DM-IL) equipped with a CCD digital camera (Nikon DS-Fi1).

Cultures were maintained in the same culture plate for 40 days. During this period weakly attached cells and spheres were sequentially identified and removed by gentle plate agitation. The suspension obtained each time was carefully pipeted and cells were placed into new plates with the same medium and culture conditions previously described.

*c) Immunocytochemistry procedure:*

Cultured cells were subjected to immunocytochemistry testing to detect any signs of maturation. For that purpose antibodies against nestin (1:200; MAB353, Chemicon-Millipore), vimentin (1:100; Boehringer Mannheim GmbH), glial-fibrillary-acidic protein (GFAP, 1:100; Novocastra) and 200kD neurofilaments (1:100; Novocastra) were used.

After fixation (4% paraformaldehyde buffered with 50 mM sodium borate, pH 9.5, 10 mins), cells were washed with phosphate buffered saline (PBS) (0.1M, pH 7.4, 15 mins). To prevent non-specific peroxidase-endogeneous reaction, cultures were incubated in a solution of $H_2O_2$ (Merck) (1% in PBS, 15 min). After PBS washing, cells were then incubated in a PBS solution containing 0.3% Triton-X (TX-100; Sigma) and 30% horse serum solution (30 mins). Primary antibody was then applied overnight in PBS with 5% horse serum and 0.3% TX-100 at 4ºC. Samples were washed in PBS and the secondary biotinylated antibody was added (1:200; in

PBS with 2.5% horse serum, 60 mins). Cells were then incubated in avidin-biotin peroxidase complex (1:100; Vectastain ABC kit, Vector Laboratories; 60 mins) and the immunoreactivity was finally evidenced by 3.3'-diaminobenzidine (DAB; Sigma).

*d) Histological procedure:*

As a control of stem cells presence and location within the auditory receptor tissue 10 newborn (P0) Wistar rat cochleae were removed and histologically processed. Briefly, cochleae were fixed in a 4% paraformaldehyde with 50 mM sodium borate buffer (pH 9.5) solution. Fixed samples were dehydrated and embedded in paraffin. Paraffin sections (10μm thick) were obtained using a microtome (Mod.1130/Biocut; Reichert-Jung, Germany). Histological sections were immunostained with anti-nestin (1:200; MAB353, Chemicon-Millipore) by using the same protocol previously described for cultured cells. The samples were visualized and photographed using a photomicroscope (Leica DM-RB) equipped with a CCD digital camera (Nikon DS-Fi1).

## RESULTS

*a) Nestin-containing cells detection in the newborn rat cochlea.*

Immunocytochemical studies showed nestin-containing cells were widely distributed in the newborn rat cochlea (Fig. 1a). There was a clear difference between the territories that expressed and did not express nestin. For example, in the spiral limbus, some nestin expression was detected in the outer portion of the interdentate cell layer, but the main proportion of nestin (+) cells was observed under this layer (Fig. 1b). In the same way, the Reissner membrane (Fig. 1c) showed immunoreactivity against nestin but was limited to some cells and fibers from the mesoderm layer. Nestin expression was also observed in the stria vascularis (Fig. 1d). In this area, nestin positive cells were detected in the mesodermal region of the stria vascularis beneath the marginal cells layer (Fig. 1d). The tympanic side of the basilar membrane was another source of cochlear nestin. As indicated in Figures 1a and e, nestin was expressed by cells located along the whole basilar membrane beneath the neuroepithelium. This neuroepithelium that would develop into the organ of Corti, did not show evidence of nestin producing cells (Fig, 1f) except in some neural fibers that reached the location where the inner hair cell (IHC) was developing to innervate it (Fig. 1e).

*b) Primary cochlear cultures.*

In the first week in culture, two different populations of cells could be distinguished morphologically. Cells growing directly attached to the culture plate

exhibited a coalescent layer of flat undifferentiated cells. Another population was constituted by rounded highly undifferentiated cells that grew in isolation or formed cellular spheres (Fig. 2a and b). These isolated cells and cellular spheres proliferated whilst weakly attached to the layer of flat undifferentiated cells. This situation was maintained during the period of study. A large proliferation of both cell populations was observed without any morphologically significant sign of differentiation (Fig.2a and b).

*c) Secondary cultures of cochlear cells spheres.*

Some cell spheres were obtained from primary cultures (see above) after one week in culture. The resultant cell suspension was then reseeded into fresh culture plates. The sphere cells attached directly to the floor of the dish. The sphere cells progressively and quickly disaggregated (Fig. 2c and d). The resulting isolated cells appeared to differentiation (Fig. 2c). Early these cells were round, ovoid or cylinder-like and then promptly showed sprouting-like processes similar to developing neuroectodermal cells (Fig. 2d). All of these differentiation-like signs were observed without adding any growth factor or differentiation promoter to the culture medium.

*d) Immunocytochemical studies on cell cultures.*

The nestin expression was detected in undifferentiated cells and cellular spheres growing in primary cultures (Fig. 3a). Nestin expression was also identified in flat cells of the monolayer in relationship with some appearance of neural

morphology (Fig. 3a). Some isolated cells and spheres also exhibited GFAP and vimentine expression (Fig. 3c, d and e). In secondary cultures, all the sphere-derived cells expressed nestin in the first 24-48h after seeding, but the positive-immunoreaction was weaker than that of the primary cultures (Fig. 3b). Two weeks later, these sphere-derived cells were found highly differentiated and some of them weakly expressed GFAP (Fig. 3f), vimentin (Fig. 3g) or 200kD neurofilaments (Fig. 3h).

## DISCUSSION

Previous studies have demonstrated that stem cells can be found within the mammalian auditory receptor. These cochlear stem cells can be evidenced by detecting the expression of the intermediate filament nestin (Malgrange et al. 2002; Lopez et al. 2004; Lou et al. 2007). In P0 rat auditory receptor, nestin expression can be detected in some conspicuous areas such as: the spiral limbus, the Reissner membrane, the stria vascularis, the basilar membrane and under developing sensory hair cells (Fig. 1).

As has been described previously, nestin expression has been considered a good indicator of the presence of developing neurons and/or stem cells. This fact can be extrapolated to the mammalian organ of Corti to determine the nature of the cochlear stem cells. Probably the most easily recognizable nestin (+) cells observed in the rat cochlea were in the developing spiral ganglion type I neurons that made contact with the developing IHC (Fig. 1e). But the description of the nature of nestin-producing cells outside the organ of Corti must be different because the rest of the cochlear nestin-expressing territories are not neural territories.

The distribution of nestin in the P0 rat cochlea can be understood when appreciating the embryological origin of the cochlear tissues (Torres and Giraldez, 1998). The inner part of the cochlear duct is derived from the otic placode, ergo ectodermal tissue. This includes the Kolliker/Corti organ, the marginal cells layer of the stria vascularis, the Reissner membrane inner layer, the interdentate cells layer of the spiral limbus and the spiral ganglion neurons derived from delamination from the

otic cup. With the exception of spiral ganglion neurons fibers of which must express nestin since it is a neuron-developing marker. None of the cochlear ectodermic structures demonstrated immunoreactivity against nestin. Therefore the presence of nestin in the rest of cochlear territories could suggest that the origin of cochlear nestin-producing cells is not ectodermal but mesodermal. In fact, nestin immunoreactivity can be observed in the intermediate cells of the stria vascularis' intermediate layer (Fig. 1a & d) which is derived from the neural crest. Our hypothesis indicates that the spiral limbus, the vestibular side of the Reissner's membrane and some of the neurons of the cochlear spiral ganglion could contain stem cells that could also derive from neural crest.

In culture conditions, these cells showed self renewal and a high proliferation rate that are typical characteristics of stem cells. However, during culture some stem cells spontaneously differentiated. It was previously reported that stem cell spheres grow well in suspension culture conditions and, when cultured in "adherent" conditions, the stem cells disaggregate from the spheres and start the differentiation process (Lou et al. 2007). If nestin is a good biomarker of a developing neuron then nestin immunodetection in these primary cultures might indicate that the deepest flat cellular monolayer could contain developing neurons (Fig. 3a). This fact has been previously supported by studies in which these flat cells have been identified in retinal cultures and characterized as neuroectodermal cells (Moyer et al. 1990). The expression of other markers such as GFAP and vimentin found in the primary cultures could also suggest the neuroectodermal origin of these cells.

In secondary cultures, cellular spheres disaggregated and showed signs of

differentiation. This spontaneous differentiation results in the expression of nestin 24-48hrs after reseeding. Even though, it was reported that nestin expression disappeared after 72hrs in culture (Lou et al. 2007). This fact could be related to the positive progression of neuron differentiation. Two weeks after reseeding, secondary cultures cells still proliferated and the sphere formation was sporadic. Only some cells remained undifferentiated, the majority of cells exhibiting morphological signs of differentiation. These cells showed a scarce immunostaining with antibodies against GFAP, vimentin or neurofilaments (Fig. 3). The addition of growth factor (EGF, IGF or FGF2) to the culture medium improved proliferation of stem cells, production of cellular spheres and the expression of some typical markers such as myosin VIIA (Malgrange et al. 2002; Li et al. 2003; Rivolta et al. 2006). In spontaneous non-induced differentiation a weak expression of cell markers was found.

In conclusion, these results demonstrate that cochlear stem cells exhibit long-term survival in our culture conditions without the addition of growth factors. During culture, stem cells preserve proliferation and self-renewal abilities in both primary and secondary cultures. Stem cells, especially in secondary cultures showed evidence of spontaneous differentiation into neuroectodermal cells. However, spontaneous differentiation can also be considered to be an indicator of possible teratogenicity of these cells. The removal and culture of any stem cell increases their tumorigenicity (Knoepfler, 2009). However, the first step of *in vitro* amplification of these cells is necessary. Further research is required to determine a better understanding of spontaneous differentiation, which cells are being obtained and possibilities of differentiation after living animal cochlear implantation.

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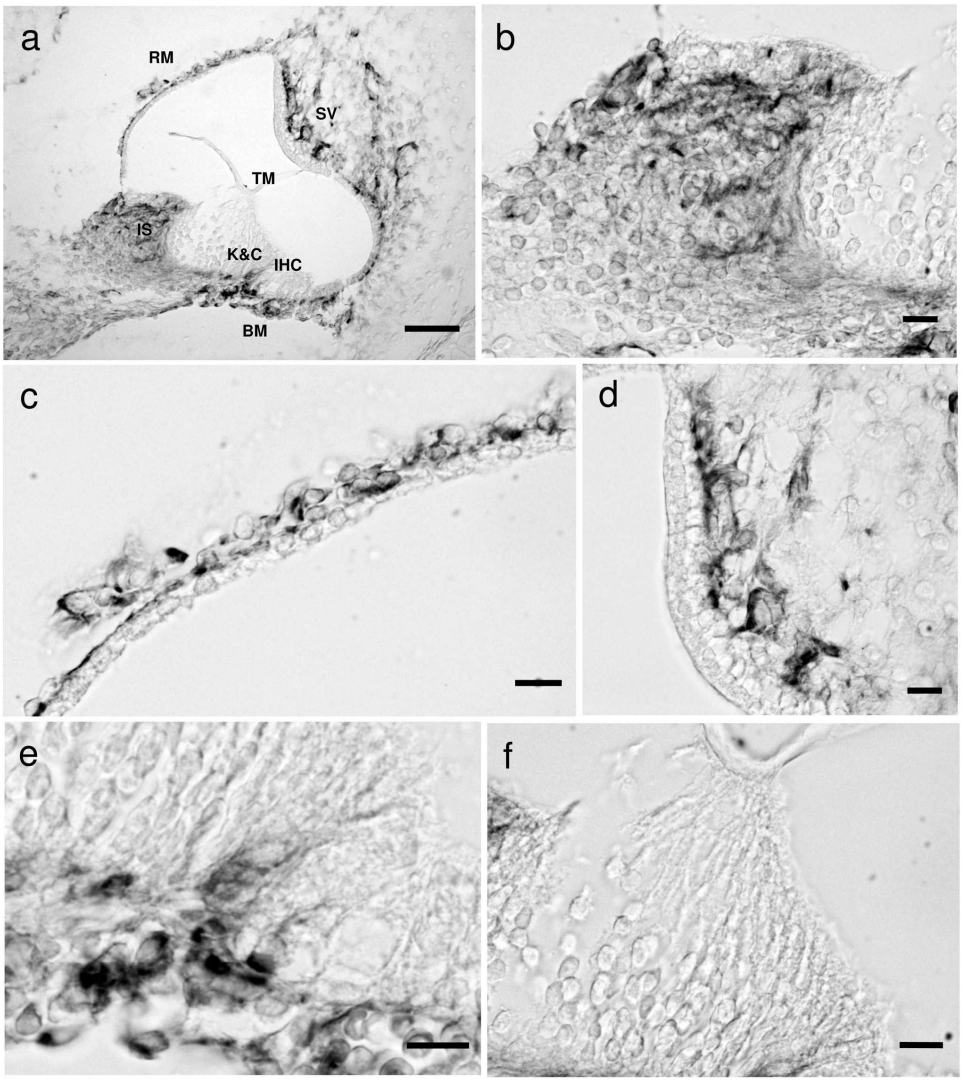
## FIGURE LEGENDS

**Figure 1**: Immunocytochemical detection of nestin in the newborn rat cochlea. a) The nestin-producing cells can be found widely distributed in the newborn rat cochlea such as spiral limbus (SL), Reissner's membrane (RM), stria vascularis (SV), basilar membrane (BM) and under immature inner hair cells (IHC). b) Spiral limbus area (SL): Nestin expression (black arrows) was observed under the interdentate cell layer (ID) and in the outer portion of this layer (star). c) Nestin expression was found in the vestibular side of the Reissner membrane (black arrows (ScV: Scala Vestibuli; CD: Cochlear Duct). d) In the stria vascularis, nestin (black arrows) was detected in some cells below the marginal cells layer (M). e and f) In the basilar membrane, nestin expression was observed in its tympanic side (ST: Scala tympani). No expression of nestin was detected in the Kolliker/Corti organ with the exception of some spiral ganglion fibers (white arrow) that will innervate the future inner hair cell (IHC). *Scale bar: a: 50μm; b-f: 10μm.*

**Figure 2**: a-b) Inner ear stem cell spheres. A subpopulation of isolated undifferentiated cells, probably stem cells (asterisks), can be observed growing over the deepest cellular monolayer in cultures of 3 (a) and 31 (b) days old. c-d) Stem cell spheres culture derived from 38 days old cultures. c) Few hours after the spheres' culture, the cells leaved the attached spheres (dotted circle) and begun the differentiation (white arrows). d) Dendritic-like processes could be observed in the differentiating cells (white arrows). *Scale bar: a-c: 50μm; d:*

*20μm*.

**Figure 3**: Immunocytochemical studies in the inner ear cultured cells. a-b) In vitro immunocytochemical detection of nestin. a) The 15 days old cultures of inner ear cells showed immunoreactivity to nestin in some grades, being the strong immunoreaction for the neuron-like differentiated cells (black arrows). Some neuro-sprouted processes (black asterisks) can be observed. The inner ear stem cells showed positive immunoreactivity to nestin (white arrowheads) that can be already observed in isolated and differentiating stem cells from cultured cell spheres (b). Inner ear stem cell and their cell spheres already showed immunoreactivity to GFAP (c-d) and vimentin (e). In cultures from isolated stem cell spheres were detected some differentiated cells that expressed GFAP (f), vimentin (g) and 200kD neurofilaments (h) (black arrowheads). *Scale bar: 50μm except b: 20μm*.

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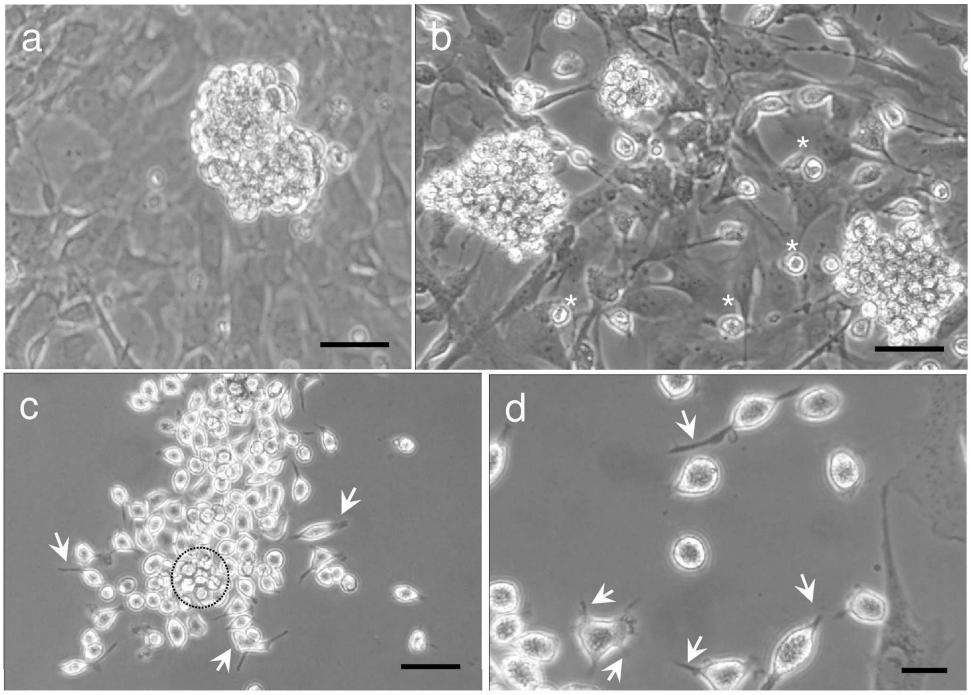


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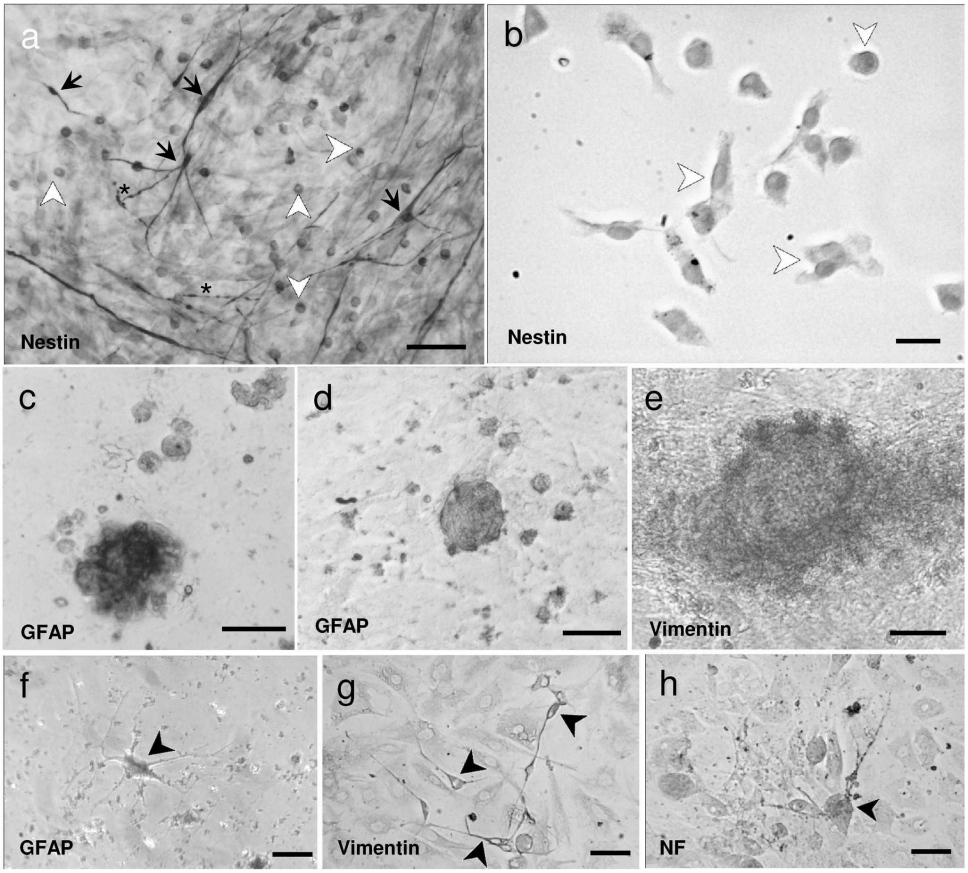


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