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## Spontaneous Association of Glial Cells With Regrowing Neurites in Mixed Cultures of Dissociated Spiral Ganglia

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### Abstract

Evidence from developmental and regeneration studies of the cochlea and other tissues give reason to hypothesize a role for non-neural cells in the growth and regeneration of cochlear spiral ganglion nerve fibers. We examined the spontaneous associations of regrowing neurites and non-neural cells in mixed cultures of dissociated newborn mouse spiral ganglia. After 7 days *in vitro*, non-neural cells formed a confluent layer in the culture well. Regrowing neurites grew atop this layer, forming non-uniform patterns that were similar to those formed by endogenously expressed laminin-1, entactin and integrin  $\beta 4$ , but not fibronectin or tenascin. In cultures grown for 42 hours and maintained in three different growth media, all regrowing neurites were preferentially associated with spindle-shaped non-neural cells. The spindle shaped cells incorporated BrdU in culture and were immunoreactive for the proteins S100, laminin-1, laminin-2, Sox10, P75 and connexin29 but negative for fibronectin and GFAP. These cells existed in the culture within a much larger, general population of fibronectin positive cells. Immunolabeling of fixed cochleas from neonatal mice localized Sox10, P75 and connexin29, to peripheral nerve bundles. The observed expressions of protein markers and the bipolar, spindle shape of the neurite-associated cells indicates that they are derived *in vitro* from the original Schwann or satellite cells in the ganglion or spiral lamina. The spontaneous and preferential association of neurites in culture with mitotic Schwann cells highlights the potential contribution neurite-Schwann cell interactions may have in promoting the growth and regrowth of damaged spiral ganglion neurons in the cochlea.

### Keywords

cochlea; regeneration; neurite; guidance; Schwann; *in vitro*

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## INTRODUCTION

In the mammalian peripheral nervous system, Schwann cells contribute to the mechanisms that underlie neuronal survival, growth and regeneration both *in vivo* and *in vitro* (Fallon, 1985; Kleitman et al., 1988a; Kleitman et al., 1988b; Seilheimer and Schachner, 1988; Meyer and Birchmeier, 1995; Chen et al., 2005; Morris et al., 2006). The neurite-promoting effects of Schwann cells on nerve fibers are mediated by the expression of trophic factors as well as by the expressions of cell surface and extracellular matrix proteins that are permissive for neurite growth (Kleitman et al., 1988a; Kleitman et al., 1988b; Seilheimer and Schachner, 1988; Letourneau et al., 1990; Schneider-Schaulies et al., 1990; Hansen et al., 2001; Wanner and Wood, 2002; Wanner et al., 2006b). The positive effects of Schwann cells on neurite growth are exerted mainly by immature, mitotic cells that have not yet begun to myelinate the nerve process (Domeniconi et al., 2002; Domeniconi and Filbin, 2005). After nerve damage, however, mature, myelinating Schwann cells are able to backtrack along their differentiation program to a more immature state that will again support neurite growth (Cheng and Zochodne, 2002).

In the cochlea, the role of non-neural cells during spiral ganglion neurite growth and their potential for aiding in neurite regeneration has not been closely examined. In adult cochleas, Schwann cells normally myelinate the peripheral nerve fibers of Type I spiral ganglion neurons along their length until they reach the habenula perforata, after which the fibers continue to grow unmyelinated into the epithelial region. In a mouse model in which Schwann cells are absent from the inner ear (erbB2 null mice), the developing cochlea displays nerve fiber projection deficits that are consistent with the idea that Schwann cells are necessary for the development of the correct, organized nerve fiber patterns in the organ (Morris et al., 2006).

In the present work, we have used cell culture of the dissociated newborn mouse spiral ganglion to examine the spontaneous associations of regrowing neurites with non-neural cells.

## EXPERIMENTAL PROCEDURES

### Animals

Newborn and postnatal day (P) 1 and P2 mice, CD-1 strain (Charles River Laboratories, Wilmington MA, USA) were used for preparation of cultures. Mice aged P3 and P9 were used for immunohistology. The care and use of the animals in this study were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of Northwestern University. Every effort was made to minimize the suffering of research animals and to limit their numbers.

### Reagents

**Cell culture**—Brain derived neurotrophic factor (BDNF) and neurotrophic factor 3 (NT3) (Promega, Madison, WI, USA; final 8 ng/ml); Fetal bovine serum – FBS (Sigma-Aldrich, St. Louis MO, USA; purchased and subsequently heat inactivated, final 10%); N2 Supplement (Invitrogen, Carlsbad, CA, USA); Leukemia Inhibitory Factor (LIF; Sigma-Aldrich, 50 ng/ml final); Bone morphogenetic protein 4 (BMP4; R&D Systems, Minneapolis, MN, USA; final 25–50 ng/ml); Bromodeoxyuridine (BrdU; BrdU labeling and detection kit II, Roche Applied Science; 1:1000); poly-D-lysine (Sigma-Aldrich P7280, 100µg/ml); laminin (Sigma-Aldrich, L2020, 10µg/ml); murine fibronectin (Invitrogen, 10µg/ml); human-tenascin C (Chemicon, now Millipore, Billerica MA; 10µg/ml);

**Immunolabeling**—mouse anti- $\beta$ III tubulin (TuJ1; 1:500–1:1000; Covance, Berkeley, CA); rabbit anti- $\beta$ III tubulin (Covance, Berkeley, CA; 1:5000); rabbit anti-mouse fibronectin (1:5000; Chemicon AB2033); rat anti-entactin (1:2500; Chemicon MAB 1946); rat anti-laminin (1:500, Chemicon MAB 1914); rat anti-mouse CD104 Integrin  $\beta$ 4 chain (1:250; Pharmingen clone 346-11A); peroxidase labeled (Fab')<sub>2</sub> of either rat anti-mouse IgG (1:100), donkey anti-rabbit IgG (1:500), donkey anti-rat IgG (1:500), and alkaline phosphatase labeled (Fab')<sub>2</sub> of donkey anti-rabbit IgG, (1:1000) all from Jackson Immunoresearch (West Grove, PA); Vectastain DAB staining kit and Vector Blue alkaline phosphatase substrate kit (Vector Laboratories Burlingame, CA); DAKO Glycergel and DAKO Fluorescent mounting medium (DAKO Cytomation, Carpinteria, CA); Alexafluor 594 labeled goat anti-mouse or anti-rabbit IgG (1:2000) and Alexafluor 488 labeled goat anti-rabbit IgG (1:2000), goat anti-rat IgG (1:500), or goat anti-mouse IgG (1:2000), and nuclear yellow, all from Invitrogen (Carlsbad, CA); rabbit anti-Sox10 (1:5000, kindly provided by Dr. Michael Wegner, Institut für Biochemie, Universität Erlangen-Nürnberg; (Stolt et al., 2003)); rabbit anti-connexin29 (1:100, Zymed/Invitrogen); rabbit anti-p75 (1:500, Chemicon); rabbit anti-myelin basic protein (Biotrend); rabbit anti-S100 (Sigma); rabbit anti-GFAP (Dako Cytomation); rabbit anti-P0 (gift of Dr. Marie Filbin, Hunter College, NY).

## Cell Culture

Cultures of dissociated spiral ganglia were prepared as previously described in detail (Whitlon et al., 2006; Whitlon et al., 2007). Cultures were maintained with three different medium additions: Control cultures contained BDNF, NT3, 10% FBS and N2 supplement. LIF cultures additionally contained Leukemia Inhibitory Factor; BMP4 cultures replaced LIF with Bone Morphogenetic Protein 4. After two days in culture, medium was changed every other day. For BrdU labeling, BrdU was added to wells two hours after plating and maintained throughout the culture period. Cultures were routinely plated on poly-D-lysine/laminin coated 96 well plates (Whitlon et al. 2007; Whitlon et al. 2006) or on glass or plastic culture slides (Lab-Tek). For some experiments, laminin was replaced by fibronectin or tenascin.

## Immunolabeling

**Immunocytochemistry:** Both enzyme-linked and fluorescent methods were used to label cultures as detailed in Table I. Enzyme-linked procedures were used with cultures in 96 well plastic tissue culture plates. Cells for immunofluorescence were cultured on glass or plastic (Nunc, Lab-Tek) multiwell slides. Neurons were identified using either a mouse or rabbit antibody directed against the neural  $\beta$ III tubulin. **Immunohistochemistry** Cochleas were embedded and sectioned at 15  $\mu$ m as described (Whitlon et al., 2001). After air drying for 2 hours, the slides were treated with 0.1% Triton X-100 in TBS for 2 hours at room temperature in a humid chamber. After rinsing in TBS, the slides were blocked for 1 hour in TBS containing 5% BSA/10% normal goat serum. The primary antibody (anti- $\beta$ III tubulin) was then added in 5% BSA/10% normal goat serum/0.1% Triton X-100 and incubated overnight at 4°C. After washing the slides in TBS, the secondary antibody was applied (Alexafluor 488 or 594 labeled goat anti-rabbit IgG or goat anti-mouse IgG; 1:2000) 0.1% Triton X-100 for 4 hours at room temperature in the dark. After washing in TBS the slides were again blocked for 1 hour and the second primary antibody was added and maintained overnight at 4°C. After washing in TBS, the second secondary antibody was added in 0.1% Triton X-100 for 4 hours at room temperature. The slides were washed with TBS and treated for 5 minutes with a .0001% solution of nuclear yellow, washed again in TBS and mounted in DAKO fluorescent mounting medium.

## Digital Photography

Cultures and tissue sections were photographed with a Nikon DXM1200 camera attached to a Zeiss Axioscope microscope under phase, partial Nomarski or fluorescence optics. Digital processing of images was limited to reorientation of photographs, converting to grayscale, resizing and contrast enhancement with either Adobe Photoshop CS or Metavue software.

## RESULTS

Spiral ganglion cultures maintained for 5–7 days generated a layer of non-neural cells that covered the entire growth surface. The overall pattern of neurite growth atop this surface was non-uniform (Fig. 1A). In some regions of the well, neurons extended long neurites that sometimes grew singly (Fig. 1A, arrow), sometimes grew in bundles (Fig. 1A, double arrow), and sometimes completely bypassed areas of underlying cells (Fig. 1A, asterisk). This behavior suggested that the underlying surface of non-neural cells supporting this neurite growth might also be non-uniform. To determine if the underlying cells elaborated extracellular matrix or cell surface proteins in a non-random pattern, similar cultures were immunolabeled for laminin-1 (Fig. 1B), entactin (Fig. 1C), integrin  $\beta 4$  (Figure 1D), fibronectin (Fig. 1E) or tenascin (Fig. 1F). The patterns formed by laminin, entactin and integrin  $\beta 4$ , but not fibronectin or tenascin, were filamentous in appearance and were similar to that created by the growth paths of the neurites. At this stage, fibronectin labeling is nearly uniform in the dish, while tenascin expression forms a patchy pattern of immunostained islands of cells. Double label of a 5-day culture shows that neurites co-localize with cells in the culture that are laminin-1 positive (Figure 2A).

The association of non-neural cells with regrowing neurites was next examined at an earlier time after plating (42 hours), when the protein coated dish was partially free of cellular covering and the individual neurites were shorter and more easily followed. We questioned whether differences in neuronal morphology, in substrate proteins or in medium composition would result in neurites growing unaccompanied on the substrate coating of the dish. It was shown previously (Whitlon et al., 2006; Whitlon et al., 2007) that when dissociated spiral ganglion is cultured on a poly-D-lysine/laminin substrate under control conditions (see methods), the 42 hour surviving neurons comprise a mixture of three main morphological types: monopolar, bipolar, and neurite-free. Inclusion of LIF in the culture medium increases neuronal survival. The resulting neuronal population has a higher percentage of bipolar neurons and longer neurites than in control cultures. On the other hand, inclusion of BMP4 in the culture medium, while also increasing survival, generates a population with a higher percentage of monopolar and neurite-free neurons, and the neurites are shorter than in control cultures. In the present study, dissociated spiral ganglia were cultured with control, LIF and BMP4 medium on dishes coated with laminin or with fibronectin or tenascin, proteins that are known to support neurite growth from other types of neurons (Rigato et al., 2002; Yong et al., 1988). On all substrates LIF and BMP4 increased survival and produced the same differences in neuronal morphology as described above for laminin growth. In all types of cultures regardless of protein substrates, composition of morphologic types, or growth medium, neurites grew only in association with non neural cells, usually spindle-shaped, rather than directly on the protein coated surface of the culture well (Examples in Figs. 1G–1L).

To determine whether the spindle shaped cells were those that were originally plated or were derived from the plated cells by mitotic division, cells were cultured in the presence of BrdU. The spindle shaped cells incorporated BrdU during the culture period, demonstrating that they were derived from mitotic division *in vitro* (Figure 2B).

The fairly ubiquitous distribution of fibronectin in the older cultures from Fig. 1E, raised the question of whether during the early stages, regrowth of the nerve fibers was indifferent to the fibronectin positive regions or whether they showed a preference for regions devoid of fibronectin positive cells. Cultures were double labeled with antibodies directed against neurons (TuJ1) and against fibronectin. At 42 hours in culture, most of the cells in the well were positive for fibronectin. Nonetheless, regrowing nerve fibers could rarely be seen in association with fibronectin expressing cell types (Figs. 2C–2F). The growth of nerve fibers and the paths of the fibronectin negative cells were so strongly linked that the association was preserved even when the growth paths made abrupt curves and bends (Fig. 1K; Fig 2C, 2D, double arrows). These observations indicate that given a choice between fibronectin positive and negative cells, neurites associate with fibronectin negative cells.

Based on their spindle shaped morphology, incorporation of BrdU in culture, lack of fibronectin labeling and tight association with neurites, we hypothesized that these were cells derived from Schwann or satellite cells of the original preparation. To determine the identity of the spindle shaped cells, we immunolabeled cells with antibodies directed against proteins that label Schwann cells in the cochlea or other regions of the nervous system – the transcription factor Sox10, the gap junction protein connexin29, the low affinity NGF growth factor receptor P75; S100; laminin-2; GFAP; or myelin basic protein (Britsch et al., 2001; Jessen and Mirsky, 2005; Tang et al., 2006). We triple labeled the cultures for the neuronal marker  $\beta$ III tubulin, one of the Schwann cell markers above, and a nuclear visualization dye. At 42 hours in culture, no cells were positive for GFAP or myelin basic protein (data not shown).

Fig 2G–H and fig 3A–E demonstrate the expressions of Schwann cell markers S100 (Fig. 2G), Laminin-2 (Fig. 2H), Sox10 (Fig. 3A,B,D), connexin29 (Fig. 3C) and P75 (Fig. 3E) in neurite-associated non-neural cells. Nuclear labeling in Fig. 3A (the same microscopical field as Fig. 3B) suggest a variety of cell types displaying different nuclear morphologies. Fig 3B shows a merged view of immunolabeling for Sox10 (green) and  $\beta$ III tubulin (red). Sox10 is expressed only in a subset of the nuclei shown in Fig. 3A. The red,  $\beta$ III tubulin-positive neuron extends its neurite preferentially over the Sox10 positive cells. In other cultures, the spindle shaped cells were labeled with antibodies directed against connexin29. Fig. 3C shows the field of nuclei of the cells in the culture (blue) and the small subset of cells that express connexin29 (green) that are spindle shaped and lie along the path of the TuJ1 positive nerve fiber (red). Figures 3D and 3E show older cultures, 5 days *in vitro*, and demonstrate that a field of cells that express Sox10 (Fig. 5D) or P75 (Fig. 5E) underlie the growing neurites.

To validate the Schwann cell markers *in vivo*, we show that in histologic cross sections of radial bundles in the P3 or P9 cochleas, P75 (Figs 3F,G), Sox10 (Figs 3H,I), and connexin29 (Figs 3N,O), label the same regions as the myelin markers P0 (Figs 3J,K) and myelin basic protein (Figs 3L,M.).

## DISCUSSION

The association with and possible dependence of neurite growth on non-neural cells has not been well studied in the cochlea. Here we examined the relationships between regrowing auditory neurites and non-neural cells *in vitro*. We approached the problem with a little used method – that of plating dissociated tissue without further purification of cells - and relied on immunocytochemical labeling for cellular characterization. After spiral ganglia and surrounding tissue were dissociated and plated, the cells were permitted to find their own cellular associations among the mixed cell types. In this “choice” experiment, in which all cell types from the region of the spiral ganglion were included and different matrix proteins

were supplied as potential growth substrates, all regrowing neurites preferentially associated with a particular subclass of spindle-shaped, non-neural cells, which this work further characterized and identified.

Finding that the spindle-shaped, neurite associated non-neural cells lacked fibronectin immunoreactivity and incorporated BrdU *in vitro*, we hypothesized that they were derived in culture from the original Schwann or satellite cells within the cochlear ganglia and spiral lamina. However, Schwann cells have not been extensively studied in the cochlea (Rio et al., 2002; Tang et al., 2006; Hurley et al., 2007) and there have been even fewer characterizations of cochlear Schwann cells *in vitro* (Hansen et al., 2001). A small number of Schwann cell markers validated in other regions of the nervous system (Zorick and Lemke, 1996; Jessen and Mirsky, 2005)– such as P75, connexin29, S100, myelin basic protein, Sox10 and P0 – have also been demonstrated in the cochlea by immunolabeling or *in situ* hybridization (Gestwa et al., 1999; Knipper et al., 1999; Watanabe et al., 2000; Coppens et al., 2001; Tang et al., 2006; Hurley et al., 2007), but not all of these are specific to Schwann cells in this organ. For example, P75, S100 and Sox10 have also been localized to cells in the cochlear epithelium and/or spiral ligament (Foster et al., 1994; Gestwa et al., 1999; Knipper et al., 1999; Watanabe et al., 2000). These cell types do not contaminate our cultures because both of these regions were carefully dissected away before dissociating cells. In contrast, connexin29 immunoreactivity is expressed strongly only by Schwann or satellite cells in the cochlea (Eiberger et al., 2006; Tang et al., 2006). It is interesting that in intact cochleas, only weak GFAP labeling is observed in the spiral ganglion or spiral lamina, even at older ages (Rio et al., 2002) since GFAP is a later marker for Schwann cells in the sciatic nerve (Mirsky et al., 2008). Strong expression of GFAP is also a marker for CNS astrocytes (Bignami et al., 1972), and its absence in our cultures indicates low, if any, contamination by astrocytes. Taken together, the P75, Sox10, laminin-1, laminin-2 and connexin29 labeling of our cells, their mitotic division *in vitro*, and their lack of GFAP or fibronectin labeling, indicates that the neurite associated cells in our cultures descended from Schwann or satellite cells in the original dissociated preparation of the spiral ganglion and surrounding tissue.

In 42 hour dissociated cultures grown on poly-D-lysine/laminin coated plastic multiwell dishes, neurites have such a strong preference for the spindle shaped, fibronectin-negative Schwann cells that they rarely associate with the extracellular matrix protein coating of the culture dish (laminin, fibronectin or tenascin) or with fibronectin positive cells. A similar association is detected on matrix coated glass, but there seem to be fewer cells lining the nerve fibers at the time of assay, although in the absence of electron microscopy, it is difficult to say whether fine processes from the Schwann cells extend beneath the entire length of the neurite. The present work does not answer the question of which comes first in the progression, neurite extension or Schwann cell association. At the times we assay the cultures, 42 hours, 5 and 7 days, we rarely, if ever, see growth cones or the ends of nerve fibers devoid of contact with Schwann cells. Such a question is better answered with time-lapse studies than with the strictly defined assay times used here. This work also raises, but cannot presently answer the question of the derivation of the spindle shaped cells. Are the progenitors in contact with the neuron at the time of plating and able therefore to divide and travel down the growing nerve fiber; or, are the progenitors recruited to the nerve fiber?

Neurites and glial cells collaborate to permit the progression of neurite growth and regeneration (Mirsky and Jessen, 1999; Chen et al., 2005). The present results are consistent with the view that Schwann cells in these cultures, whatever their derivation, arrive at the growing tip of the neurite, thereby creating a permissive local environment for further extension of the nerve fiber. In general, during development throughout the nervous system *in vivo*, “guidance” of growing nerve fibers is thought to depend largely on the interactions

that growing nerve growth cones have with their microenvironments. The consistent association of regrowing spiral ganglion nerve fibers with Schwann cells in the culture supports the view that actual “pathfinding” *in vitro* may be an indirect process. Neurites may find Schwann cell surfaces a permissive growth environment, but the pathway they traverse seems more to do with the cues discerned by the Schwann cells than the interactions of the neurites with other elements of the culture environment. This view would be consistent with the work of Wanner et al. (2006a) who found that in developing E14 rat embryos, membranes of Schwann cell surfaces covered a constant 80% of the neuronal growth cone surface as it was nearing its target in the forelimb. It was concluded that at the nerve front it was the Schwann cell precursor, rather than the growth cone itself, that was most exposed to the extracellular environment.

In cochleas, when the hair cell targets of spiral ganglion neurons are destroyed by acoustic overstimulation, antibiotics or other drug toxicity, some of the disconnected spiral ganglion neurons die – the exact proportion differs among species (Teufert et al., 2006). The peripheral fibers of the remaining neurons degenerate, perhaps as far as to the cell soma. In humans, enough of these once bipolar neurons remain connected to the brain stem to permit the functionality of cochlear implants, which rely on electrical stimulation of the neurons or their central fibers to generate auditory information for the brain to decode. Inducing the regrowth of the peripheral nerve fibers toward a cochlear implant has been suggested as a way to decrease the power requirement and increase the frequency selectivity of the implant (Roehm and Hansen, 2005). In this regard, it is encouraging that even adult spiral ganglion neurons have the capacity to sprout to some extent after noise damage or in the presence of infused neurotrophins. In four separate studies of this limited sprouting *in vivo*, cells with the appearance of Schwann cells were detected in association with the nerve fibers (Bohne and Harding, 1992; Lawner et al., 1997; Wise et al., 2005; Glueckert et al., 2008). Further studies will be required to determine the role of Schwann cells in encouraging, supporting or directing neurite growth and regeneration in the inner ear.

Both positive effects of Schwann cells and negative or non-permissive effects of other cells play a role in sculpting the neuronal fiber patterns in nervous tissue. Having in hand an *in vitro* system in which Schwann cells and neurites interact in a microenvironment that is populated by other cells derived from the spiral ganglion will aid in the discovery of biochemical mechanisms that support spiral ganglion neurite regeneration as well as potential non-permissive mechanisms that may interfere with neurite regrowth.

## ABBREVIATIONS

BDNF	brain derived neurotrophic factor
BMP4	bone morphogenetic protein 4
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
IgG	immunoglobulin G
LIF	leukemia inhibitory factor
MBP	myelin basic protein
NT3	neurotrophic factor 3

P	postnatal day
P0	protein zero
P75	neurotrophin receptor
S100	an acidic calcium binding protein
Sox10	SRY-related high-mobility-group box 10 transcription factor
TBS	Tris buffered saline

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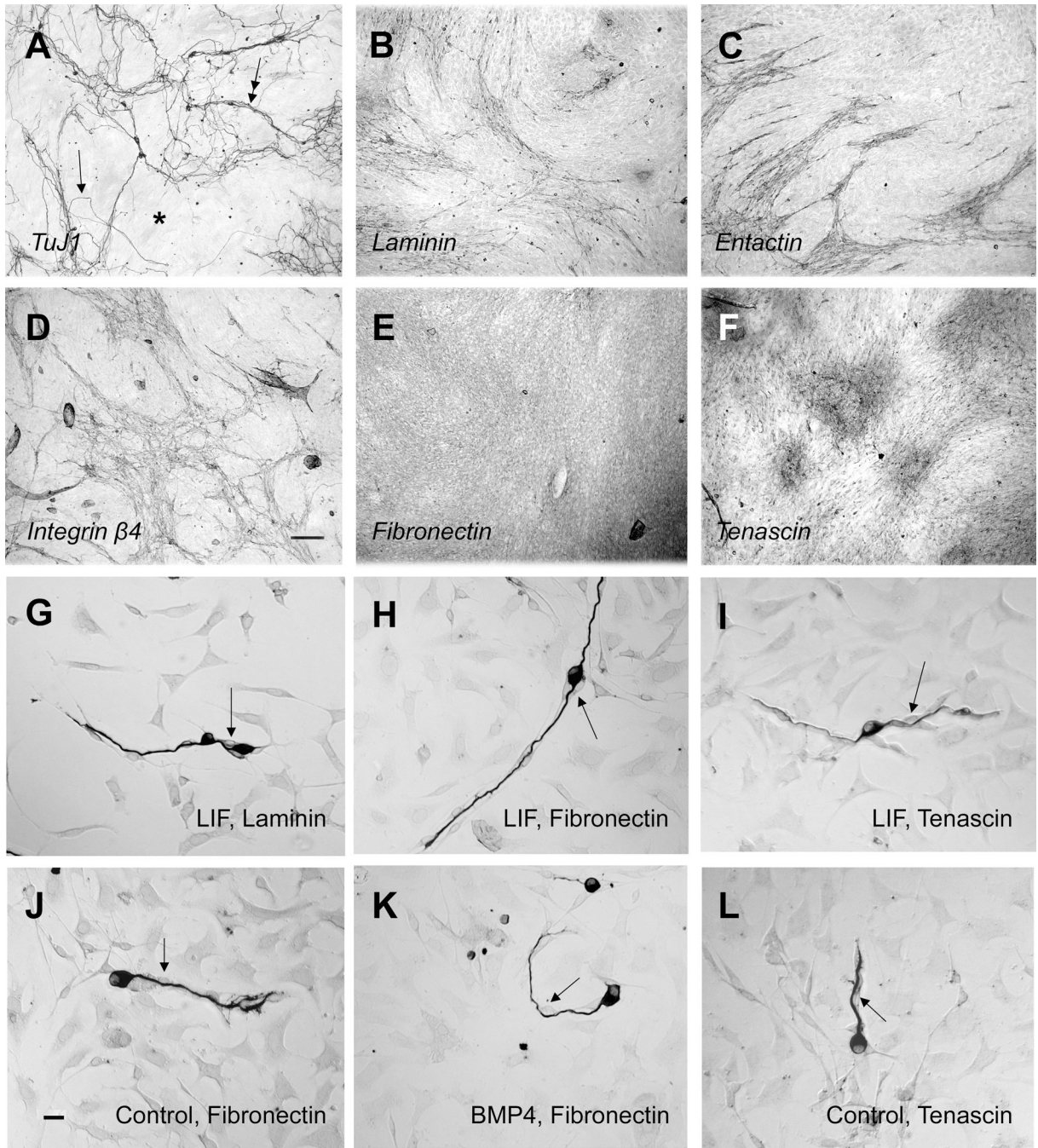
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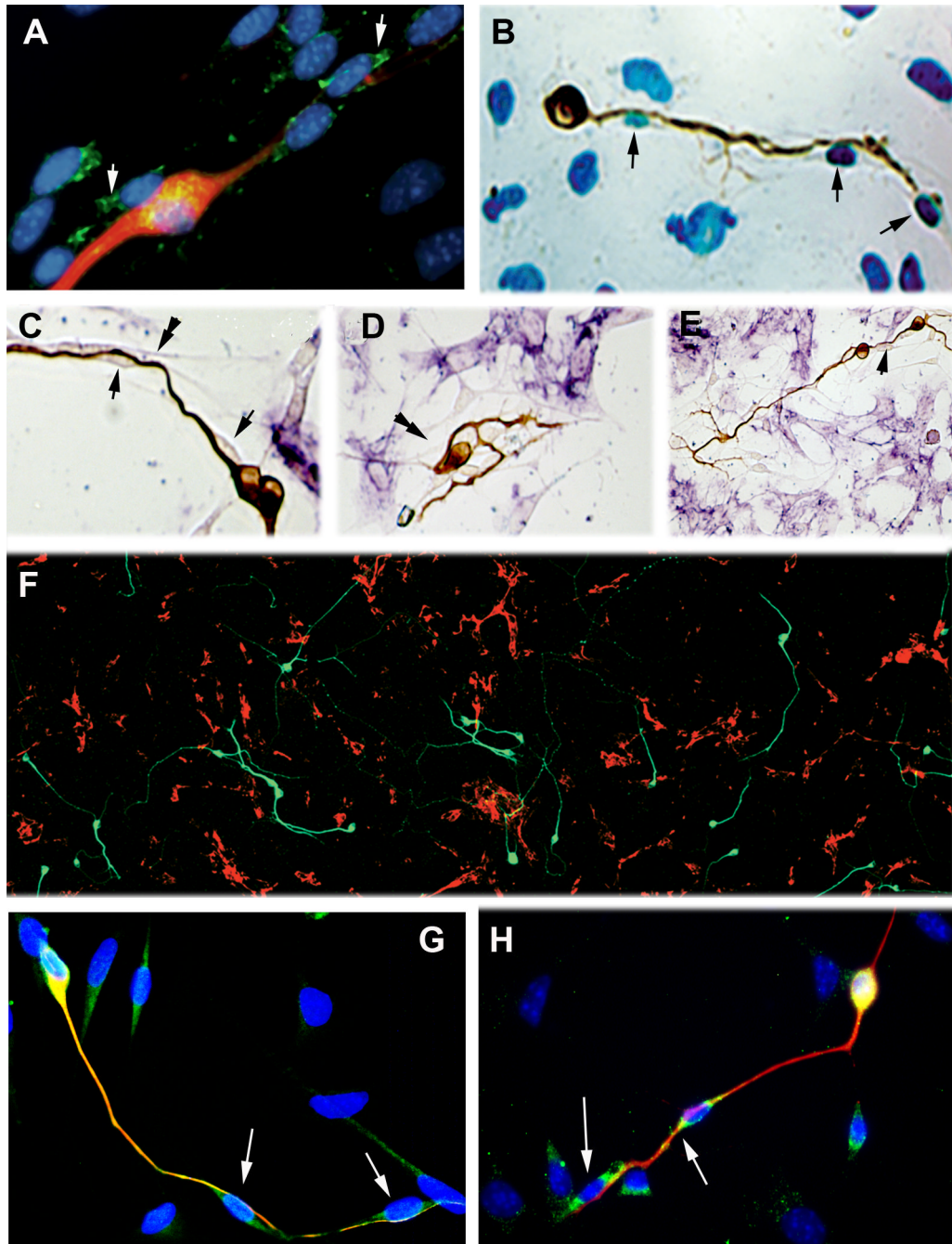
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**Figure 1.**

A–F. Examples of 7-day, confluent cultures immunolabeled as shown in italics. G–L. Examples of 42 hour cultures maintained under different growth factor and substrate conditions and immunolabeled with TuJ1 to highlight neurons. (A). The nerve fiber pattern in this longer term culture is non-uniform. Fibers extend in some regions and avoid others (asterisk), growing singly (arrowhead) but also, often growing in bundles (double arrowhead) atop the underlying non-neural cells. (B–D) Endogenously generated laminin-1 (B), entactin (C) or integrin  $\beta 4$  (D) form filamentous, non-uniform patterns in the culture; (E) Fibronectin is expressed fairly homogenously across the nonneural cells. (F) Tenascin immunoreactivity is observed in islands of stain across the culture well. (G–L) Under all

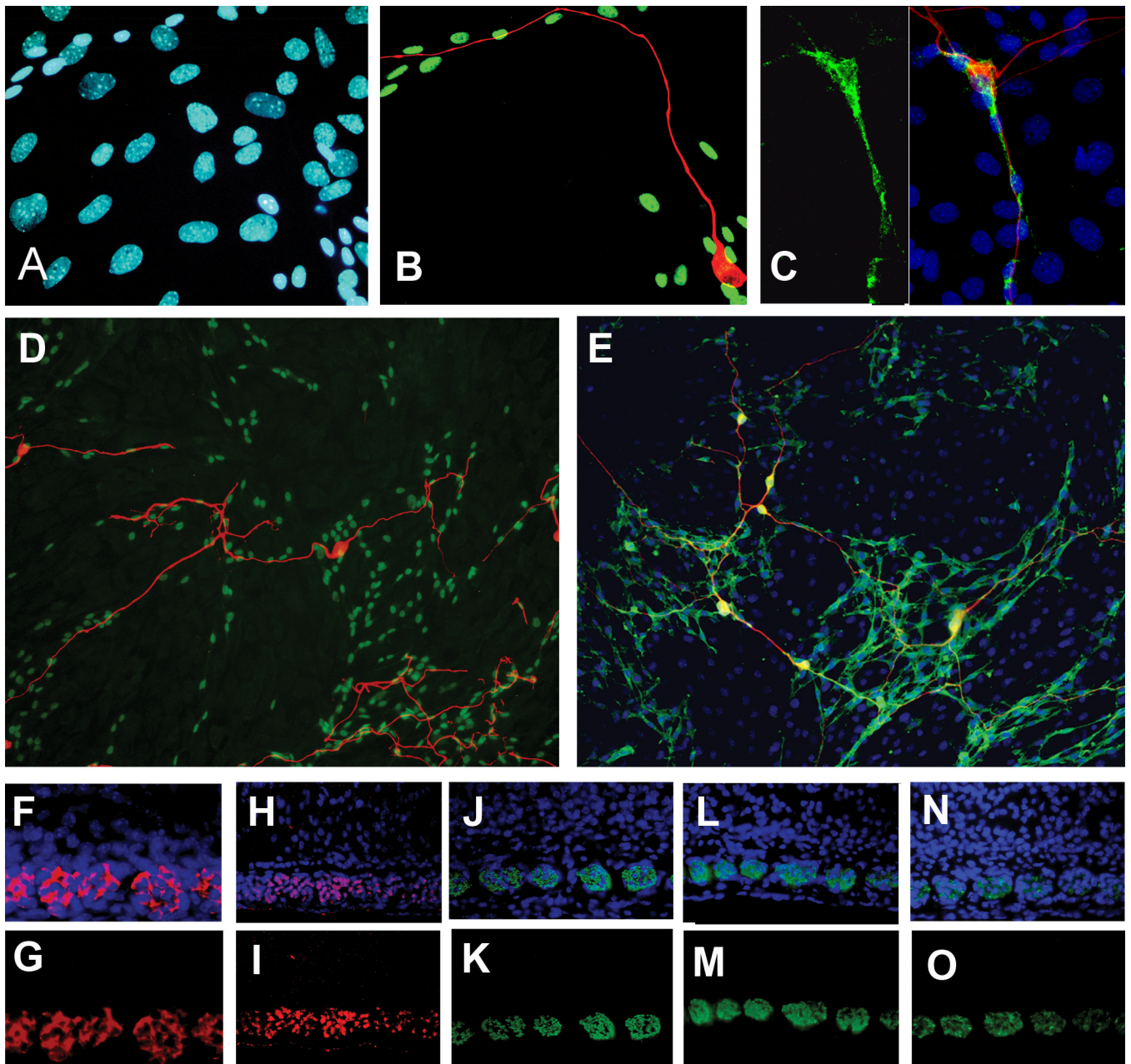
tested conditions (control, LIF or BMP4 medium and laminin, fibronectin or tenascin coating of the culture well), neurites grow only in association with a type of non-neural cell that is usually spindle shaped (arrows). Neurites and spindle-shaped cells retain their associations even throughout abrupt changes in the direction of growth (see K).



**Figure 2.**

42 hour cultures. (A) Immunolabeled for neurons (TuJ1, red), Laminin-1 (green). Nuclei (blue). The red neuron is associated with cells that express laminin-1. The cells at the lower right are not immunoreactive for laminin-1. (B) Cells lining the regrowing neurites (brown) incorporate BrdU (blue) into their nuclei (arrow) indicating that they were produced by mitotic division in culture. (C–E). Double immunolabeled for fibronectin (purple) and TuJ1 (brown). Neurites grow on a class of non-neural cells (arrows) that do not express fibronectin. The association of the neurites with these cells is maintained even when the direction of growth changes (double arrowhead). (F) Overview, double immunolabel for fibronectin (red) and TuJ1 (green). Only rarely can TuJ1 labeled neurites be seen to cross

regions of fibronectin immunoreactivity. (G) Cells lining the neuron (TuJ1, red) are S100-positive (green). Merge (yellow) shows that neurons also express S100. Nuclei, blue. Cells in the upper right do not express S100. (H) Laminin -2 positive cells (green) line the TUJ1-positive neuron (red). Nuclei, blue. Merge (yellow) demonstrates laminin-2 around the neuronal soma.



**Figure 3.**

A–B, 42 hour culture, same microscopical field. (A) Blue nuclei depict the locations of all cells in the field. (B) Green nuclei show that a subset of cells that express Sox10 lie along the growth path of the neuron (red). (C) Left, connexin29 labeling. Right, same microscopical field, a neuron (red) associates with spindle shaped cells that express connexin29 (green) amid a field of blue nuclei. No other cells in the field are positive for connexin29; (D). 5-day culture. Red, TuJ1 (neurons), green, subset of nuclei expressing Sox10. (Nuclei of confluent cell layer are unlabeled in this image for clarity). Nerve fibers grow only on the field of Sox10 positive cells. (E) 5-day culture. Red, TuJ1 (neurons), Green P75, Yellow, merge. Nuclei, blue. Nerve fibers grow only on the region of cells expressing P75. Neurons are immunopositive for P75. (F–O): Cross sections of the radial bundles in fixed cochleas. (F,G) Postnatal day 3. Same microscopical field: blue, nuclei; red,

P75. (H,I) Postnatal day 9. Same microscopical field: blue, nuclei; red Sox10; pink, nuclei expressing Sox10. (J,K) Postnatal day 9. Same microscopical field. blue, nuclei; green, P0. (L,M) Postnatal day 9. Same microscopical field. Blue, nuclei; green, myelin basic protein. (N,O) Postnatal day 9. Same microscopical field. Blue nuclei; green, connexin29.



**Table I**

Outline of procedures for single and double labeled enzyme linked labeling and double fluorescent labeling of cultures.

	Enzyme-Linked		Fluorescent
	Single	Double	Double
Fix	4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 × 1 hr @ RT	Same	4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 × 15 min
Wash	TBS	TBST	TBS
Quench	TBS/methanol/30% hydrogen peroxide/water (5:1:0.4:3.6) × 10 min @ RT	Same	Skip
Wash	TBS	TBST	Skip
Solubilize	0.3% Triton × 2 hr @ RT	Same	Same
Wash	<i>0.01% ovalbumin in TBS or TBST</i>	Same	TBS
Block	<i>0.1% ovalbumin in TBS or 10% NDS+2% NFD in TBS × 1 hr @ RT</i>	10% NDS+2% NFD in TBS for 1 hr @ RT	5% BSA/10% NGS in TBS × 1 hr @ RT
1° antibody	<i>Diluted in 0.03% Triton in TBS or in 10% NDS+2%NFD in 0.3% Triton in TBS. 3 days @ 4°C</i>	TuJ1 in blocking solution overnight @ 4°C	TuJ1 or rabbit anti-βIII tubulin diluted in 5% BSA+10% NGS +0.3% Triton overnight @ RT
Wash	<i>0.01% ovalbumin in TBS or in TBST</i>	TBST	TBS
2° antibody	<i>Diluted in 0.3% Triton in TBS or 2% NFD in 0.3% Triton in TBS × 4 hours @ RT</i>	TBS containing 2% NFD and 0.3% Triton × 4 hours @ RT	Alexafluor conjugated antibody diluted in 0.1% Triton in TBS × 4 hours @ RT; dark
Wash	<i>TBS or TBST</i>	TBST	TBS
Visualization	Vectastain DAB staining kit	Same	Skip
Mounting	2–4 drops DAKO Glycergel per well	Skip	Skip
Wash		Skip	TBS
Block		10% NDS+2% NFD in TBS for 1 hr @ RT	5% BSA + 10% NGS in TBS × 1 hour @ RT
Second 1° Antibody		Diluted in 10% NDS+2% NFD +0.3% Triton × overnight @ 4°C	Diluted in the blocking solution × overnight @ RT
Wash		TBST	TBS
Second 2° Antibody		AP labeled antibody diluted in TBS containing 2% NFD and 0.3% Triton × 4 hours @ RT	Alexafluor conjugated antibody diluted in 0.1% Triton in TBS × 4 hours @ RT; dark
Wash		TBST, then TBS	TBS
Visualize		AP substrate kit, 15 minutes; @ RT; dark	
Wash		Tris-HCl, pH 8.2, then TBS	
Mount		2–4 drops Glycergel	
Visualize nuclei			0.001% nuclear yellow × 5 min @ RT
Wash			TBS
Mount			DAKO fluorescent mounting medium

Abbreviations: NDS, normal donkey serum; NFD, non-fat dry milk; RT, room temperature; Triton, Triton-X-100; BSA, Bovine serum albumin. Words in italics refer to procedures used only with mouse monoclonal antibodies.