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Research Article

Olfactory ensheathing cell phenotype following implantation in the lesioned spinal cord

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Abstract. Although olfactory ensheathing cells (OECs) are used to promote repair in the injured spinal cord, little is known of their phenotype in this environment. In this study, using quantitative reverse transcriptase-polymerase chain reaction RT-PCR, expression of neuregulin-1 mitogen/survival factors and the axonal growth regulator Nogo was quantified in OECs and compared with other non-neuronal cells. Their expression was also compared with OECs which had previously been encapsulated in a porous polymer tube and implanted into the injured spinal cord. Similar to astrocytes and fibroblasts, OECs expressed various neuregulin subtypes including neu differentiation factor, glial growth factor and sensory and motorneuron-derived factor. Implanted OECs upregulated neu differentiation factor and secreted neuregulin, but downregulated expression of all other variants. OECs and oligodendrocytes expressed Nogo-A, -B and -ABC and were immunopositive for Nogo-A protein. The Nogo-A protein in OECs was found to be cytoplasmic rather than nuclear or cell surface associated. Unlike oligodendrocytes, OECs expressed Nogo-66 receptor (NgR) mRNA. Implanted OECs upregulated Nogo-A and -B, but downregulated Nogo-ABC and NgR.

Key words. Olfactory ensheathing cells; neuregulin-1; Nogo; quantitative RT-PCR; spinal cord injury.

Olfactory ensheathing cells (OECs) are well known for their unique ability to support olfactory axonal outgrowth throughout life [1–3]. Implantation of these cells into the lesioned spinal cord can enhance neurite outgrowth or remyelination of injured axons and promote functional recovery [4–7]. Although there is an increasing body of evidence to suggest that OECs produce neurotrophic factors [8–10] and cell adhesion molecules [11–16] that may play a role in their growth-promoting activity, there has been little progress toward understanding what constitutes regenerative OEC biology.

Because OECs are an extremely plastic population in vitro and in vivo both in terms of their morphology and antigenic profile [12, 17–19], the lesion environment may induce OECs to exhibit characteristics associated with their role in regeneration. Similarly, any changes in OEC phenotype brought about by exposure to such an environment could affect cell behavior. To investigate this possibility, we quantitatively examined the mRNA profile of neuregulin-1 (Nrg-1), a growth-promoting class of proteins, and Nogo a regulatory family of proteins in OECs after implantation into a rat model of spinal cord injury.

Nrg-1 isoforms are involved in the survival, proliferation and/or differentiation in OECs [20–22], Schwann cells [23, 24], oligodendrocytes [25–27] and astrocytes [28, 29]. The Nrg-1 gene products arise by alternative splicing and have been classified as type-I neu differentiation factor (NDF)/heregulin or acetylcholine receptor-inducing activity (ARIA), type-II glial growth factor (GGF) and type-III sensory and motorneuron-derived factor (SMDF)

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[30–32]. Each of these transcripts can be subject to further alternative splicing events within specific regions, creating huge diversity among mRNA variants.

The Nrg-1 transcripts were of particular interest due to their role as mitogens and survival factors for OECs [20–22]. OECs are also known to express Nrg-1 mRNA and protein [33, 34]. Similarly, OECs possess functional erbB receptor tyrosine kinases [22, 35], suggesting that they may respond to Nrg-1 signals in both a paracrine and autocrine manner. Furthermore, the finding that cultured OECs express an array of Nrg-1 transcripts [34] makes this gene an excellent candidate for analysis of the potential variation in OEC phenotype brought about by exposure to the injury environment.

Nogo is a central nervous system (CNS) myelin-associated regulator of neurite outgrowth and plasticity which has been localized on the surfaces of oligodendrocytes (OLGs). The Nogo gene encodes three major protein products termed Nogo-A, -B and -C that arise by alternative splicing and promoter usage [36]. Nogo has a 66-amino acid residue luminal/extracellular domain similar to that found in members of the Reticulon protein family [37]. The Nogo-66 domain was found to be inhibitory to axonal extension and fibroblast spreading [38]. Its activity is mediated by the high-affinity receptor NgR, a glycophosphatidylinositol (GPI)-linked protein that can also act as a receptor for other CNS myelin inhibitory factors such as myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) [38, 39]. Although OECs have thus far been generally associated with growth-promoting molecules, of interest would be to investigate whether they express axon inhibitory molecules. Given that OECs are now being used in clinical trials [40], a thorough understanding of their cell biology will have major implications for their therapeutic use.

In this study, we examined the expression profile of the neuregulin-1 and Nogo gene in OECs in culture and after implantation into a defined injury model using real-time quantitative RT-PCR and immunohistochemistry. Expression of these molecules was quantified in OECs and compared with that of astrocytes and fibroblasts.

Materials and methods

OEC cultures

All procedures involving animals were approved by the Animal Experimentation Ethics Committee of the University of Tasmania and are consistent with the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes.

Primary OEC cultures were prepared as described previously [10, 41]. Briefly, ten 1- to 2-day-old hooded Wistar rat pups per culture were anesthetized and their olfactory bulbs dissected. The nerve fiber layer of each bulb was removed and digested in 0.09% trypsin and 0.25% collagenase, then filtered through nylon mesh (80-µm pore size). After centrifugation for 10 min at 500 g, the cells were resuspended and plated into a 25-cm^2 flask. Cells were cultured in modified Eagle's medium D-valine modification (Sigma), containing 10% dialyzed fetal calf serum (FCS) and 1% penicillin-streptomycin-fungizone solution at 37 °C, 5% CO_2. Contaminating fibroblasts were removed by addition of cytosine arabinofuranoside (2.5×10^{-7} M). To enhance the proliferation of OECs, 125 µg/ml of bovine pituitary extract (BPE) was added to the cultures. Once sufficient confluency was obtained, cells were passaged to a new flask by trypsinization with 0.25% trypsin in Hank's balanced salt solution (HBSS). Cells were then grown for 3 days or until they reached confluency. To determine the purity of the cultures, cells were grown on round 12-mm-diameter coverslips at a density of 1×10^4 per coverslip and immunolabeled with $p75^{NTR}$ and glial fibrillary acidic protein (GFAP). Olfactory ensheathing cells were estimated to be 91% $p75^{NTR}$/GFAP positive.

Astrocyte and oligodendrocyte cultures

Primary astrocyte and OLG cultures were prepared from the cortices of 2-day-old hooded Wistar rat pups and purified using a modification of a previously described method [42]. In brief, the whole brain was dissected out and the meninges, cerebellum and brainstem removed. The tissue was minced and triturated through a flam-polished pasteur pipette, then filtered through nylon mesh (80-µm pore size). Cells were centrifuged at 500 g for 5 min and plated at a density of $1–2 \times 10^7$. Cultures were maintained in DMEM-F12 (Life Technologies) with 10% FCS and 1% penicillin-streptomycin-fungizone solution for a period of 7–10 days, following which they were shaken at 180 rpm for 6 h to remove microglia. Medium was then replaced and the cultures were shaken for a further 18 h to remove the loosely attached layer of OLGs. Astrocytes were trypsinised and plated at a density of 3×10^4 cells per 75-cm^2 flask. Cultures were maintained in DMEM-F12 with 10% FCS and 1% penicillin-streptomycin-fungizone solution until they reached confluence. To determine the purity of the cultures, cells were plated at a density of 1×10^4 per coverslip and immunolabeled with anti-GFAP. Astrocytes were estimated to be 92% GFAP positive.

Suspension of OLGs obtained after shaking the mixed glial culture was centrifuged for 10 minutes and resuspended. The cells were then plated in DMEM-F12 with 10% FCS and 1% penicillin-streptomycin-fungizone solution at a density of 3×10^4 and maintained at 37 °C overnight. The culture medium was then changed to DMEM-F12 containing 1 × G-5, 50 ng/ml tri-iodothyronine (T3) and 1% penicillin-streptomycin-fungizone solution. Cells were cultured for 10 days with media

changes every second day. To determine purity, cells were grown on coverslips at a density of 1×10^4 per coverslip. Oligodendrocytes were immunolabeled with MAB328 (Chemicon), a mature oligodendrocyte marker for which 98% of cells were positive.

Fibroblast culture

Explant cultures of fibroblasts were prepared using skin dissected from the back of 2-day-old hooded Wistar rat pups. A 1-cm^2 slice of skin was placed into a petri dish containing warmed PBS and cut into small pieces. Explants of approximately 2-mm^2 were placed into a 50-cm^2 dish and maintained in DMEM containing 10% FCS, 200 mM glutamine and 1% penicillin-streptomycin-fungizone solution. After about 7 days, cells were passaged using 0.25% trypsin/EDTA solution and centrifuged at 250 g for 10 min. The pellet was resuspended in 20 ml medium and distributed into two 75-cm^2 flasks. Cells were returned to the incubator and grown to confluence.
Brain tissue to be used for RNA extraction was dissected from 1- to 2-day-old rat pups, snap frozen in liquid nitrogen and stored at –80 °C.

Preparation of olfactory ensheathing cells for encapsulation

Upon reaching confluence, OECs were either frozen as monolayers at –80 °C for RNA extraction or encapsulated into polymer tubing for implantation into the injured spinal cord. Cells to be placed in capsules were removed from the culture flasks by trypsinization. Flasks were rinsed with HBSS three times before 0.25 % trypsin was added. The cell suspension was then centrifuged at 500 g. All supernatant was removed and the cell pellet resuspended in 5 µl of growth factor-reduced Matrigel Matrix (Becton Dickenson). An aliquot of suspension containing ~5×10^4 cells was injected into each open-ended polymer tube (Bio/Por Type F PVDF hollow fiber, MWCO: 300,000) 6–8 mm in length. Tubes were heat sealed at both ends to create closed capsules. OEC-filled capsules were then implanted into the injured spinal cords of adult male hooded Wistar rats. Control OEC-filled capsules were placed in a petri dish containing MEM D-valine supplemented with 10% dialyzed FCS and incubated for 1 week in a 5% CO_2/air incubator during which time three media changes were performed.

Spinal surgery

Lesions to the corticospinal tracts (CSTs) of adult hooded Wistar rats (300–350 g) were performed under isoflurane anesthesia maintained at 1.5–2.5% with 100% O_2 (flow rate of 0.5 l/min). The skin overlying the spinous processes of T5–T10 was incised and the underlying muscles were dissected and retracted. The spinous processes T7 and T8 were removed along with the lamina to expose the dorsal surface of the spinal cord. Fine microdissection scissors were used to make a transverse cut across the dorsum of the spinal cord. After lesioning the cord, a capsule containing either OECs or matrigel was placed on top of the lesion under the dura. The overlying muscle was sutured and the skin closed with wound clips. One week after implantation, rats were sacrificed by CO_2 inhalation and the capsules removed. Capsules were then rinsed in PBS, snap frozen in liquid nitrogen and stored at –80 °C.

Total RNA isolation and quantitation

Total RNA was isolated from cultured cells using TRIzol reagent (Life Technologies) according to the manufacturer‘s instructions. To isolate total RNA from encapsulated cells, the capsules were minced and triturated in TRIzol reagent prior to processing using a modification of the protocol described by the manufacturer. To improve the yield of RNA from encapsulated OECs, 10 µg of RNase-free glycogen (Life Technologies) was added prior to precipitation of the RNA with isopropyl alcohol to act as a carrier to the aqueous phase. Total RNA from neonatal brain tissue was extracted using the RNeasy maxi kit (Qiagen) according to the manufacturer’s specifications. RNA samples were pretreated with DNAse 1 (Sigma) to remove any genomic DNA contamination. A portion of each RNA sample was resuspended in TE buffer and quantified spectrophotometrically at A_{260}. RNA purity was determined using the A_{260}/A_{280} ratio.

Probe and primer design for the neuregulin-1 gene

Taqman quantitative RT-PCR probe/primers were designed to selectively detect different subtypes of rat Nrg-1 transcripts, as defined by Marchionni et al. [32]. The domain structure observed in different variants encoded by the human Nrg-1 gene are generated by alternate splicing of genomic exons. These variants can be classified into several subtypes (NDF/heregulin, SMDF, GGF and ARIA), based on the presence or absence of the alternately spliced exons. Since the genomic structure of the rat Nrg-1 gene is currently unknown, a strategy was devised to delineate the exon-exon boundaries (and hence, domain boundaries) of rat Nrg-1 by comparison of all known rat Nrg-1 cDNAs against the human genomic Nrg-1 sequence. Thus, all known rat Nrg-1 cDNA sequences from GenBank were aligned with the complete human gene sequence and the putative exon-exon boundaries were defined. These boundaries were consistent for all cDNAs examined ($n = 10$), indicating that this approach is robust. Identification of exon-exon boundaries allowed design of RT-PCR primers (six forward, six reverse) targeted to specific rat Nrg-1 exons (table 1). Further specificity was given by design of three Taqman probes, which specifically hybridized to the NDF/GGF2 common domain (probe 1), epidermal growth factor (EGF) common domain (probe 2) and to a separate region of the EGF

Table 1. Probe and primer combinations for amplification of Nrg-1 mRNA transcripts.

Primers and probes		Transcript class	Amplicon length
Probe 1			
Nrg-F1	Nrg-R1	GGF	106
Nrg-F2	Nrg-R1	NDF	95
Nrg-F5	Nrg-R1	NDF/GGF	72
Probe 2			
Nrg-F4	Nrg-R3	EGF domain	87
Nrg-F4	Nrg-R4	secreted	240
Nrg-F4	Nrg-R5	transmembrane	221
Nrg-F3	Nrg-R3	SMDF	149
Probe 3			
Nrg-F6	Nrg-R7	β types	147
Nrg-F6	Nrg-R8	α types	174

Nrg-1 transcript classes were identified by combining each specific probe with the forward (Nrg-F) and reverse (Nrg-R) primer combinations shown above. Amplicon length is given in base pairs. EGF, epidermal growth factor.

common domain (probe 3). In appropriate combinations as outlined in table 1, PCR specific for up to nine subtypes of rat Nrg-1 could be performed.

All probe and primer sequences were designed using the program Primer Express 1.0. The Nrg-1 and GAPDH probe/primer sets were synthesized by Applied Biosystems. Fluorogenic probes were labeled with the 5′ reporter dye 6-carboxyfluorescein (FAM) and the 3′ quencher dye 6-carboxytetramethyl-rhodamine (TAMRA).

Probe and primer design for the Nogo gene

A GenBank search was performed to identify all known Nogo sequences and to determine their regions of overlap. Three different primer sets were designed to amplify the nogo gene (table 2). The Nogo-ABC common primers amplify a region in the common C-terminal domain of Nogo-A. The Nogo-A primers identify a region within the Nogo-A-specific exon 3, and the Nogo-B primers overlap a region corresponding to the splice site that truncates the sequence. All Nogo primers were synthesised by Qiagen.

A Taqman probe and primer set for GAPDH was designed to amplify the region corresponding to nucleotides 1603–1669 of GenBank sequence accession number AF106860 producing a product with an expected size of 67 bp. The probe sequence was located between nucleotides 1630–1647.

Real-time quantitative RT-PCR

Quantitative RT-PCR for the Nrg-1 isoforms was performed using the one-step TaqMan EZ RT-PCR Kit (Applied Biosystems) according to the manufacturer's protocol. The amplification reactions were performed in a final volume of 25 µl containing ~50 ng total RNA, 2–4 mM MnOAc (depending on the isoform), 0.3 mM dATP, dCTP and dGTP, 0.6 mM dUTP, 0.10 U/µl rTth DNA polymerase and 0.01 U/µl AmpErase UNG in 1 × RT-PCR buffer. The final concentration of GAPDH forward and reverse primers was 500 nM while the probe concentration was 250 nM. The final concentration of the Nrg primers was 900 mM and the probes were 250 nM. Quantitative RT-PCR was performed using the Rotorgene 2000 (Corbett Research). Amplification conditions included 2 min at 50 °C, 30 minutes at 60 °C, 5 min at 95 °C and then run for 45 cycles at 94 °C for 15 s and 60 °C for 1 min.

RT-PCR for the Nogo isoforms was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's specifications. Each 25-µl reaction contained 50 ng total RNA, 5 mM $MgCl_2$, 1 × RT-PCR master mix, 1 × RT mix and 500 nM primers (dependant on isoform). Reverse transcription was performed at 50 °C for 30 min. Amplification reactions included an activation step of 15 min at 95 °C and were cycled at 94 °C for 15 s and 60 °C for 30 s for 30 cycles.

Data analysis

Quantitation of Nrg-1 and Nogo mRNA expression was performed using the relative standard curve method. Standard curves for each mRNA isoform including GAPDH and Nogo, or subtype class neuregulins were produced using known quantities of total RNA extracted from neonatal rat brain. For all unknown samples, the relative amount of GAPDH, Nrg-1 and Nogo isoforms was quantified by extrapolation of C_T values from the corresponding standard curve performed using the Rotorgene 5.0 software. A relative expression value for each isoform in the unknown samples was then obtained by division of that value by the specific GAPDH value. The data presented in the graphs and tables are the mean expression levels (arbitrary units) ± standard deviation (SD) normalized to GAPDH.

PCR product characterization

In addition to quantitative RT-PCR analysis, all products were analyzed by agarose gel electrophoresis on a 1.5% gel stained with ethidium bromide to ensure amplification of the appropriately sized product. No-template controls were included to identify any genomic DNA contamination. SYBR green products were subjected to melt-curve analysis to verify that all products had the correct melting temperature (T_m) and to ensure the absence of primer-dimer formation.

Immunofluorescence staining of OECs for Nogo-A and NgR

For immunocytochemical analysis, cultured OECs and OLGs were plated onto 12-mm-diameter glass coverslips at a density of 1×10^4 per coverslip. Cells were fixed with

Table 2. Primer sequences designed to analyze Nogo and the NgR in OECs.

Primers	Sequence (5′–3′)	Amplicon length (bp)	GenBank accession number
Nogo-ABC forward	ATTGTAAGCTGCTGTGTATGGATCT	89	AJ242961
Nogo-ABC reverse	ACAGCTTTCCCCGAGTCCTT		
Nogo-A forward	TGTAGTGCAGCCCTTCACAG	199	AJ242961
Nogo-A reverse	GCACATCCCTACTTCCCTCA		
Nogo-B forward	GGCTCAGTGGTTGTTGACCT	208	AJ242962
Nogo-B reverse	GGCCTTCATCTGATTTCTGG		
NgR forward	ACAACACCTTCCGAGACCTG	194	AF462390
NgR reverse	GGCAAAACGGGTAGAGGGTCA		

phosphate-buffered 4% paraformaldehyde for 15 min at room temperature. After washing three times in 0.1 M PBS (pH 7.4), the cells were incubated with DAKO protein-blocking solution. The cells were incubated with polyclonal goat anti-Nogo-A (S-19) (Santa Cruz) at a final concentration of 10 µg/ml in PBS containing 0.3% Triton-X 100 for 1 h at room temperature. The negative controls were incubated with PBS containing 0.3% Triton-X 100 without primary antibody. Cells were rinsed three times and incubated with a donkey anti-goat Alexa Fluor 594-conjugated secondary antibody (Molecular Probes) diluted 1:2000 in PBS containing Triton-X 100 for 45 min at room temperature. Cells were counterstained with 0.01% nuclear yellow and mounted onto glass slides with DAKO Fluorescent Mounting Medium. They were then examined by fluorescent microscopy using an Olympus BX50 microscope with the appropriate filter for the dye conjugated to the secondary antibody. Cells were imaged with an Olympus DP50 digital camera.

For immunocytochemical analysis of the NgR, cultured OECs on coverslips as described above were incubated with goat polyclonal anti-NgR (Santa Cruz) at a final concentration of 10 µg/ml in PBS for 1 h at 37 °C. Controls were incubated in PBS without primary antibody. Cells were then post-fixed with phosphate-buffered 4% paraformaldehyde for 15 min at room temperature. After three washes in PBS, cells were incubated with a donkey anti-goat Alexa Fluor 594- (Molecular Probes) conjugated secondary antibody diluted 1:2000 in PBS for 45 min at room temperature. Cells were counterstained with 0.01% nuclear yellow. Coverslips were mounted and examined as described above.

Results

Quantitation of Nrg-1 expression in cultured OECs

The expression profile of nine subtypes of Nrg-1 transcripts in primary cultures of OECs was compared with that of astrocytes and fibroblasts. Samples were analyzed in triplicate as described above using a TaqMan-based quantitative RT-PCR approach, and the results were normalized to GAPDH (table 3). OECs astrocytes and fibroblasts expressed largely similar subtypes of Nrg-1 transcripts while the transmembrane variant was absent in all three cell types (fig. 1). However, in addition, fibroblasts lacked GGF expression (fig. 1D). High levels of NDF transcripts were expressed by OECs compared to astrocytes and fibroblasts (fig. 1A). Moderate levels of NDF/GGF, β type, EGF common domain and secreted transcripts (fig. 1C, E, F, H) were expressed by each cell type, with OECs expressing the highest and astrocytes the lowest levels of each transcript. OECs expressed the highest levels of SMDF compared to astrocytes and fibroblasts (fig. 1B). Unlike the other transcripts, the expression

Table 3. Relative Nrg-1 mRNA levels normalized to GAPDH.

Nrg-1 isoform	OEC C	OEC CI	OEC	AC	FB
GGF	ND	ND	0.008 ± 0.0002	0.004 ± 0.0001	ND
NDF	0.123 ± 0.049	1.226 ± 0.158	4.646 ± 0.172	1.231 ± 0.182	2.658 ± 0.035
SMDF	0.024 ± 0.009	0.016 ± 0.002	0.403 ± 0.023	0.091 ± 0.020	0.058 ± 0.005
NDF/GGF	1.640 ± 0.768	0.108 ± 0.028	0.1442 ± 0.394	0.202 ± 0.054	0.459 ± 0.004
EGF domain	0.536 ± 0.287	0.079 ± 0.013	0.946 ± 0.040	0.212 ± 0.012	0.429 ± 0.101
Secreted	0.026 ± 0.016	0.112 ± 0.070	0.819 ± 0.327	0.162 ± 0.057	0.332 ± 0.165
α types	16.160 ± 3.377	4.707 ± 1.643	15.440 ± 1.609	7.423 ± 0.427	18.970 ± 3.219
β types	ND	ND	0.854 ± 0.278	0.057 ± 0.010	0.110 ± 0.048
Transmembrane	ND	ND	ND	ND	ND

Relative mRNA levels are given in arbitrary units ± SD. ND, not detected.

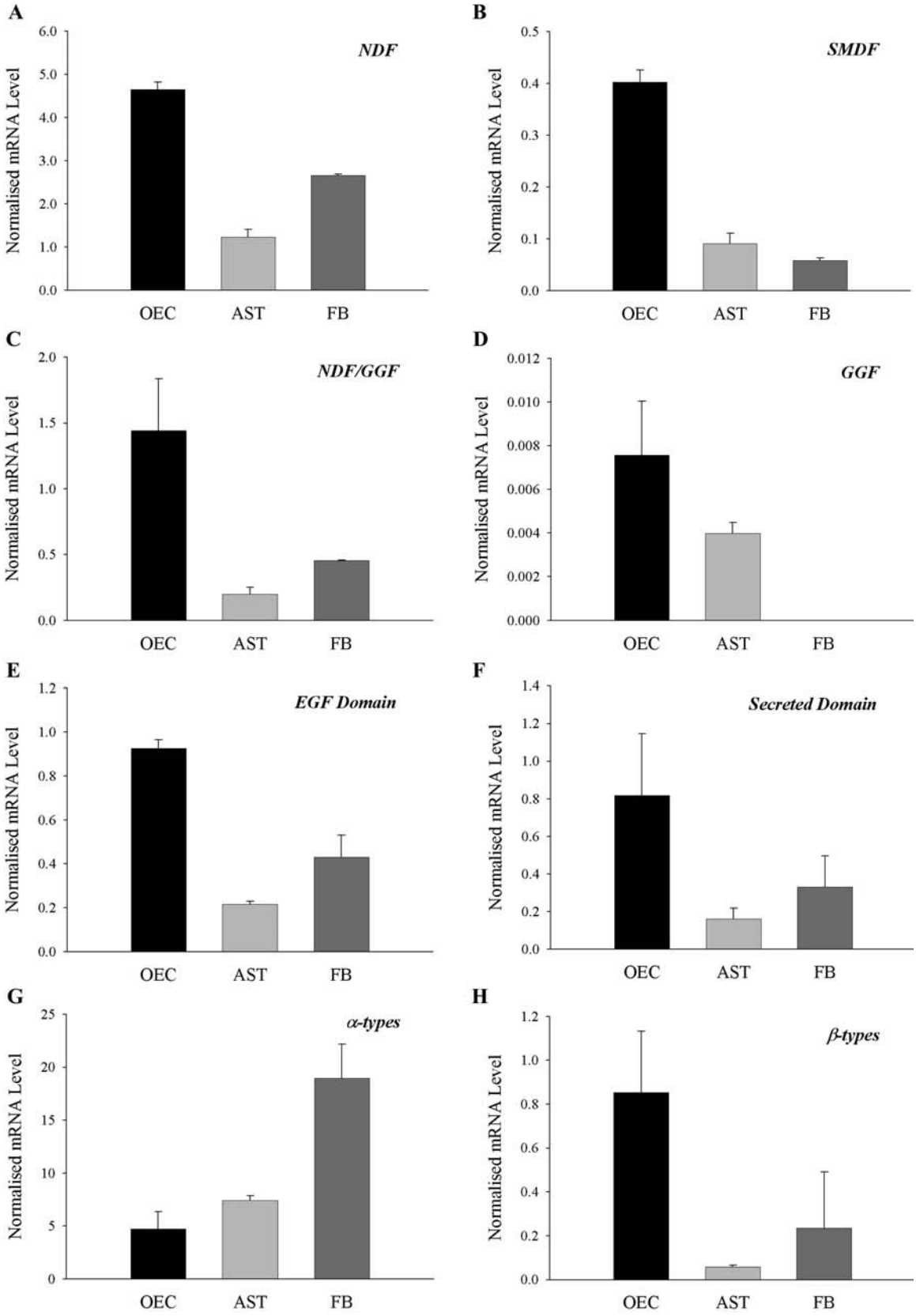

Figure 1. Expression levels of Nrg-1 isoforms in primary cultures of olfactory ensheathing cells (OEC) astrocytes (AST) and fibroblasts (FB). Cultured OECs expressed a similar profile of Nrg-1 expression as AST and FB although the levels of each transcript varied between the cell types. OECs expressed the highest levels of each transcript (*A–F*, *H*) except for the α type variant (*G*). Error bars represent the SD.

level of α type mRNA was greatest in fibroblasts, with OECs expressing the lowest levels of the transcript. No-template controls (not shown) showed no contamination with genomic DNA.

Changes in neuregulin-1 expression in encapsulated OECs

To determine whether Nrg-1 expression in OECs was affected by the in vivo environment of the injured spinal cord, quantitative RT-PCR analysis was performed on cells that were encapsulated and implanted (OEC CI) into the injured spinal cord for 1 week. For comparison, encapsulated cells were maintained for 1 week under the same culture conditions as the monolayered cells (OEC C). Samples were analyzed in triplicate for each transcript and the results normalized to the housekeeping gene GAPDH (table 3). After implantation of OECs into the injured spinal cord, only two transcript subtypes, NDF and secreted domain, were found to increase in expression level compared with control encapsulated OECs (fig. 2A, E). The NDF transcript increased by 12-fold after implantation, while the secreted domain subtype increased by 4.5-fold. The expression levels of NDF/GGF, SMDF, EGF and α type subtypes decreased after implantation (fig. 2B, C, D, F), while the transmembrane and β type subtypes were not detected in implanted or control encapsulated OECs. No-template controls showed that there was no contamination with genomic DNA (not shown).

Quantitative analysis of Nogo in cultured OECs

Expression of Nogo and its receptor NgR was examined in cultured OECs and compared with that of OLGs by quantitative RT-PCR analysis using SYBR green as the detection system (table 4). Cultured OECs and OLGs were found to express Nogo-A-, Nogo-B- and Nogo-ABC-specific mRNA isoforms (fig. 3). Oligodendrocytes expressed a higher level of Nogo-A than OECs (fig. 3A) while OECs expressed more Nogo-B and Nogo-ABC mRNA than OLGs (fig. 3B, C). Cultured OECs were also positive for the NgR (fig. 3D) which was not detected in OLGs, confirming previous reports that suggested OLGs lack the NgR [43].

Quantitative changes in Nogo in encapsulated OECs

To determine whether Nogo expression in OECs was affected by the injured spinal cord environment, OECs were injected into polymer capsules and maintained in culture or implanted into the lesioned spinal cord for one week. After encapsulation OECs retained expression of Nogo-A, Nogo-B and Nogo-ABC mRNAs (fig. 4). However, implantation into the injured spinal cord resulted in an increase in the expression level of Nogo-A (fig. 4A) and Nogo-B (Fig. 4B) and a decrease in Nogo-ABC (fig. 4C), compared with encapsulated cells maintained in culture. Unlike normally cultured OECs, encapsulated OECs lacked NgR expression whether maintained in culture or implanted into the spinal cord.

OECs express Nogo-A protein

RT-PCR analysis demonstrated that cultured OECs, regardless of whether they are encapsulated or not, as well as those encapsulated and implanted in the spinal cord, consistently expressed Nogo (figs. 3, 4). NgR mRNA was only present in unencapsulated OECs cultured in flasks (fig.3). Immunocytochemical analysis of cultured OECs from flasks revealed that the cells were immunopositive for Nogo-A protein (fig. 5A). As a positive control, cultured OLGs were also immunostained for Nogo-A (fig. 5D). Interestingly, OEC Nogo-A could only be detected after permeabilizing the cells, whereas OLG Nogo-A was detectable without need for permeabilization. Nogo-A staining in OECs was cytoplasmic rather than nuclear, consistent with the known association of the protein with the endoplasmic reticulum [37]. Although NgR mRNA was detected in normally cultured OECs, attempts to immunostain for the protein were negative.

Discussion

This study is the first to quantitatively examine gene expression changes in cultured OECs in response to implantation into the injured rat spinal cord. First, we examined the normal expression profile of Nrg-1 and Nogo in cultured OECs. For the in vivo study, OECs were encapsulated into porous polymer tubes and implanted into the injured rat spinal cord. For comparison, encapsulated OECs were kept under culture conditions for the same period of time as the implanted capsules and analyzed in the same manner.

Cultured OECs were found to express three of the major subtypes of Nrg-1 isoforms including NDF, GGF and SMDF, consistent with the findings of a previous study [34]. Our study also investigated several other splice variants including secreted, EGF, α type and β type variants, each of which was expressed by cultured OECs. In contrast to the aforementioned study, we did not detect mRNA for the transmembrane variant in OECs [34]. This discrepancy may be related to differences in the age and strain of animals used and/or to the method of culture. For example, cells used in this study were isolated from 2- to 3-day-old hooded Wistar rat pups and purified by serial passage and use of mitotic inhibitors to produce a 91% $p75^{NTR}$ positive population. Cells in the previous study were prepared from 7-day-old Sprague-Dawley rat pups and purified by fluorescence-activated cell sorting using the O4 antigen. These factors alone may have resulted in a slightly different population of OECs leading to the discrepancy in neuregulin expression observed. Another possibility is that the use of different primer sets in each

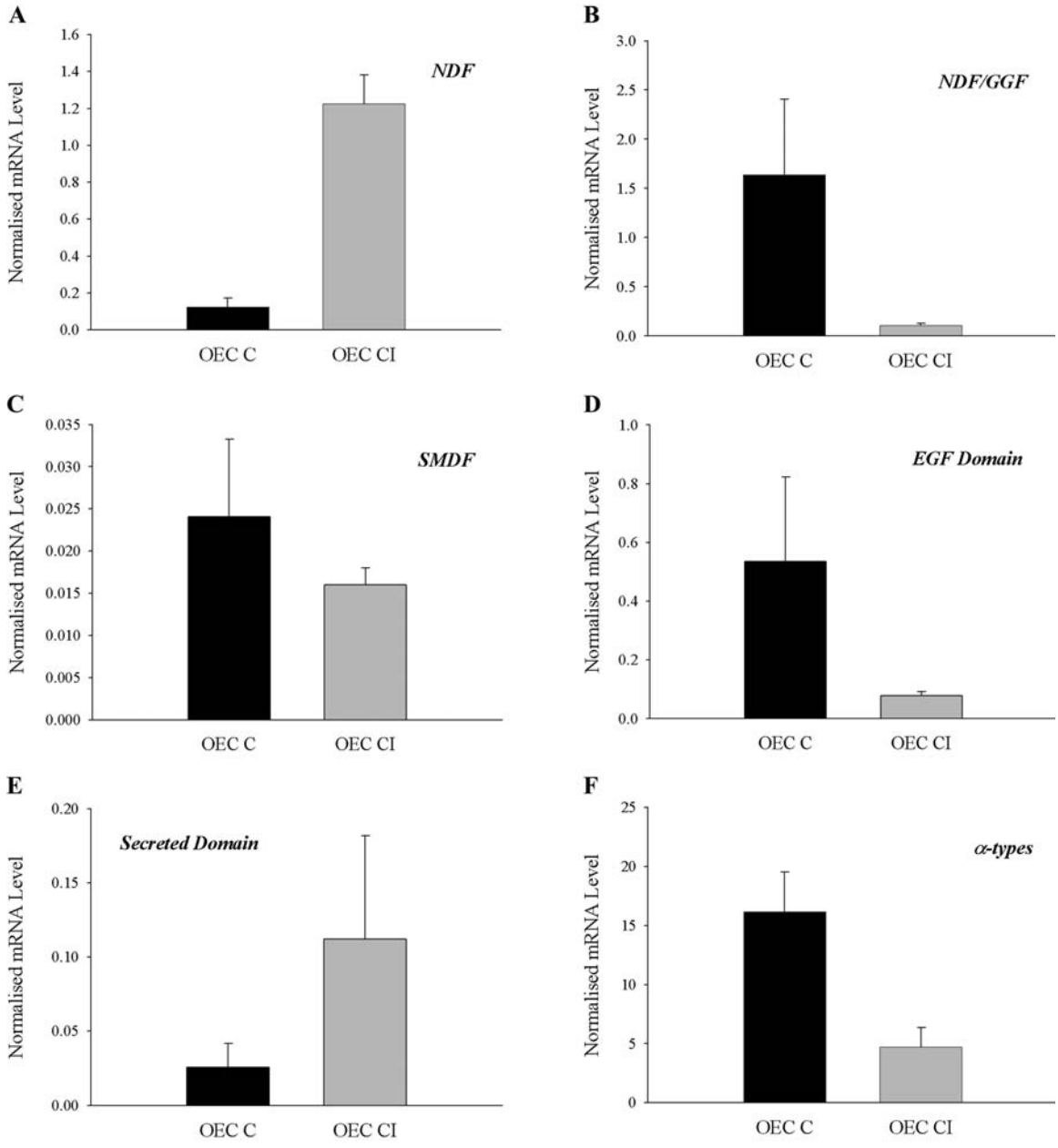

Figure 2. The effect of the injured rat spinal cord on Nrg-1 expression in olfactory ensheathing cells (OECs). Cultured OECs were encapsulated into porous polymer tubes and implanted into the injured rat spinal cord for 1 week (OEC CI), or maintained in MEM D-valine supplemented with 10% dialyzed FCS at 37 °C/5% CO_2 for 1 week (OEC C). Implantation into the injured spinal cord resulted in downregulation of NDF/GGF (*B*), SMDF (*C*), EGF (*D*) and α type (*F*) mRNA, and upregulation of NDF (*A*) and secreted Nrg-1 (*E*). Error bars represent the SD.

Table 4. Relative Nogo and NgR mRNA levels normalized to GAPDH.

Nogo isoform	OEC C	OEC CI	OEC	OLG
Nogo-A	0.015 ± 0.002	0.042 ± 0.010	0.303 ± 0.044	0.409 ± 0.070
Nogo-B	0.130 ± 0.041	2.219 ± 0.382	14.04 ± 1.018	4.79 ± 0.379
Nogo-ABC	5.016 ± 2.040	1.355 ± 0.496	0.633 ± 0.180	0.394 ± 0.207
NgR	ND	ND	0.116 ± 0.019	ND

Relative mRNA levels are given in arbitrary units ± SD. ND, not detected.

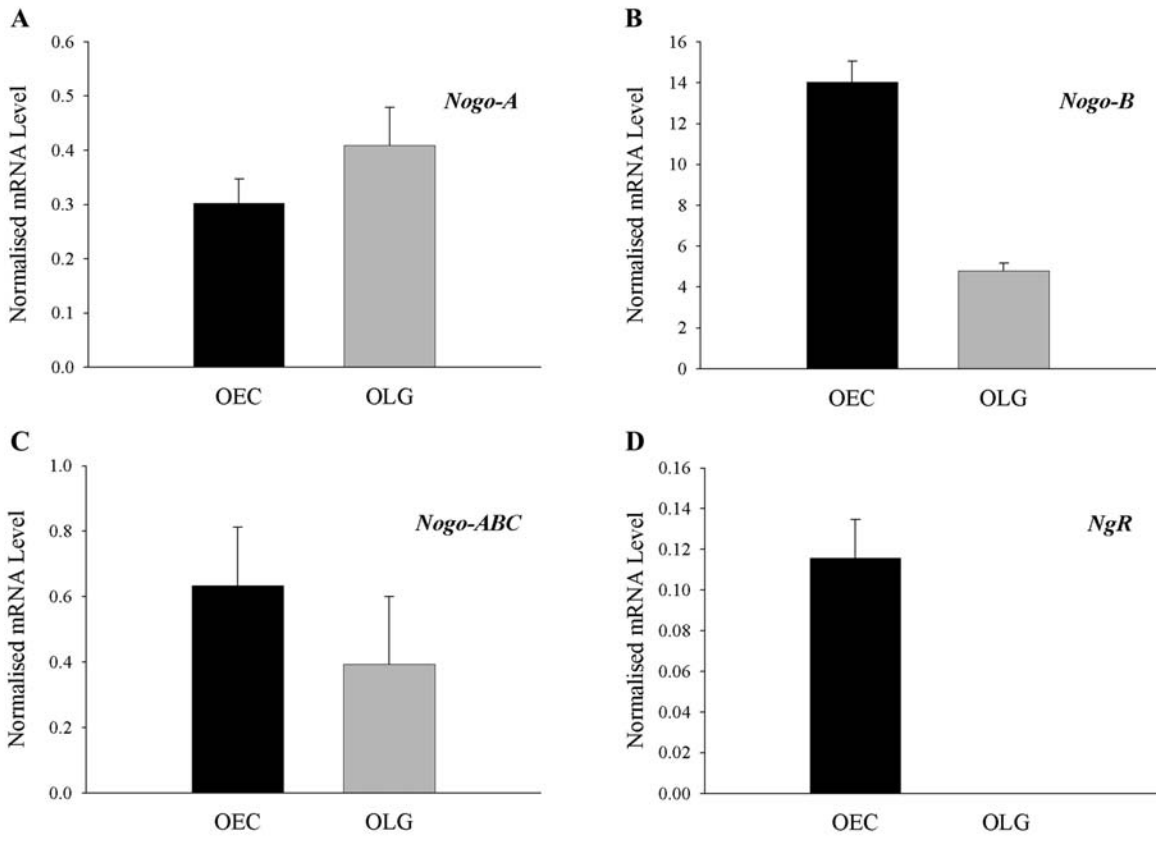

Figure 3. Nogo and NgR mRNA expression in primary cultures of OECs and oligodendrocytes (OLG). Cultured OECs expressed lower levels of Nogo-A (*A*) than OLGs but higher levels of Nogo-B (*B*) and Nogo-ABC (*C*). OECs also expressed NgR mRNA which was not present in OLGs (*D*). Error bars represent the SD.

study contributed to the apparently different result. The transmembrane primer set used here was designed to detect a single isoform. However, the primer set used by Thompson and colleagues [34] was reported to amplify both α and β transmembrane variants identified as two separate bands on the same gel. Furthermore, they report that only the α form is expressed by OECs while the β form is expressed in the cortex [34]. This may suggest that the isoform detected by our primer set was the β form. Similarly, this could explain the absence of the transmembrane variant in OECs and its presence in whole-brain RNA used in this study as a positive control. After implantation into the injured spinal cord, OECs increased expression of NDF and secreted Nrg-1. Since NDF-α and -β variants are known to be mitogens and survival factors for OECs [22], implanted OECs may increase expression of NDF to enhance their own survival and proliferation in an autocrine manner. This seems especially likely given that OECs are known to express functional erbB receptors including erbB2 and erbB4 [22]. Furthermore, our finding that OEC numbers are greatly reduced after encapsulation may also support a role for upregulation of genes involved in survival and proliferation.

Nogo-A, -B, -ABC and NgR mRNAs were detected in cultured OECs. In comparison, oligodendrocytes expressed each of the Nogo isoforms but lacked expression of the NgR, as expected based on previous studies [43]. The major inhibitory isoform Nogo-A was found to be expressed at a greater level in oligodendrocytes than OECs, while the levels of Nogo-B and -ABC in OECs were higher than in oligodentrocytes. Although there have been no published reports to date regarding Nogo expression in OECs, Nogo and its receptor NgR have been detected previously in the olfactory system [43–45]. Nogo-A and Nogo-ABC were detected by in situ hybridization in the olfactory bulb of fetal (E20) rats although no data regarding expression in neonatal or adult animals was reported [44]. The NgR is also expressed in the olfactory bulb during embryonic development and in the adult mouse, where it was localized to neurons of the mitral cell layer [43]. Although these studies do not mention OECs, they do suggest a possible role for Nogo in both the developing and adult olfactory system.

Notably the quantitative RT-PCR revealed the presence of NgR mRNA in cultured OECs in flasks but it was not detected when the cells were encapsulated either in culture

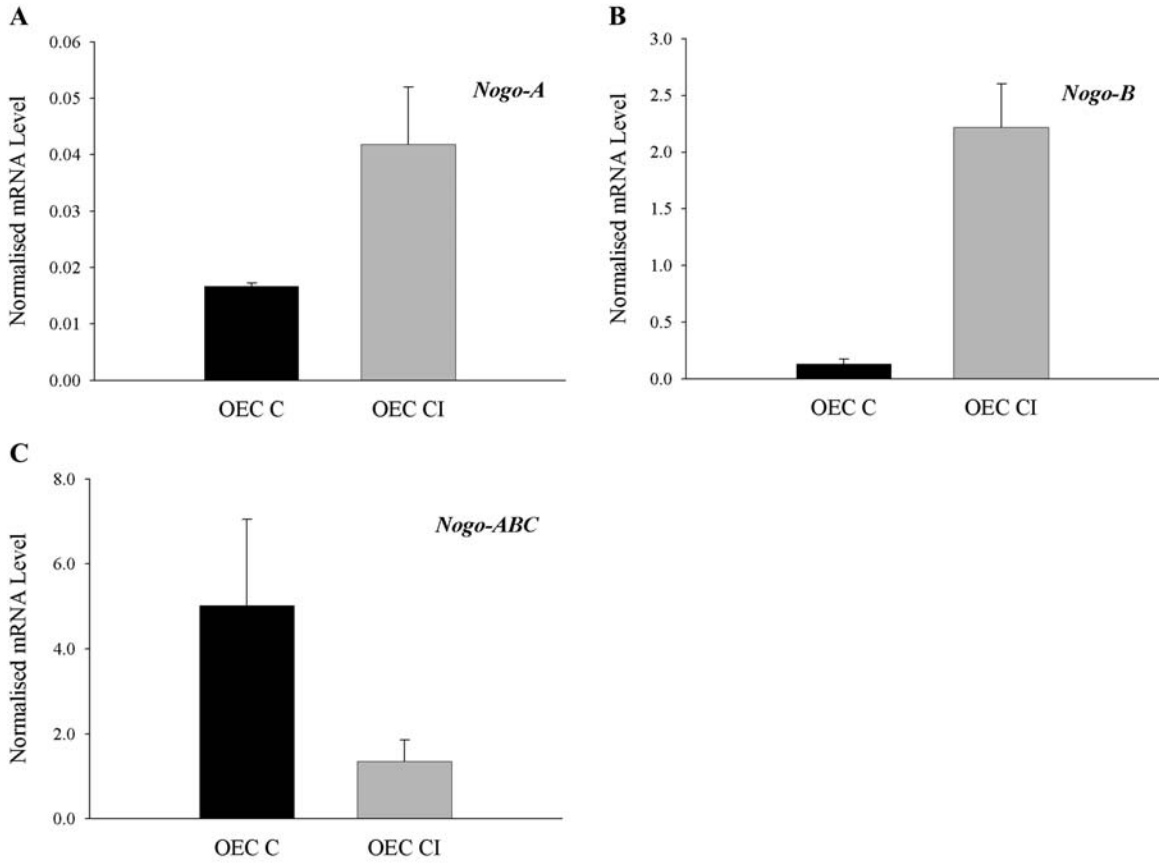

Figure 4. The effect of the injured rat spinal cord on Nogo mRNA expression in cultured olfactory ensheathing cells (OECs). Encapsulated OECs were analyzed after implantation into the spinal cord for 1 week (OEC CI). Alternatively OEC capsules were maintained in MEM D-valine supplemented with 10% dialysed FCS at 37 °C/5% CO_2 for 1 week (OEC C). After implantation into the injured spinal cord, encapsulated OECs increased expression of Nogo-A (*A*) and Nogo-B (*B*) but decreased expression of Nogo-ABC (*C*). No mRNA for NgR was detected in implanted or control capsules. Error bars represent the SD.

or when implanted. The underlying reasons for this are unclear, but from our observations based on light and scanning electron microscopy, there were distinct differences in the morphology between OECs cultured in flasks and those encapsulated. When cultured in flasks, OECs flatten and elaborate processes [19], but when placed in capsules, many of them assume a round morphology [unpublished observations]. The difference in morphology could reflect a physiological difference possibly in the expression of cell membrane-associated molecules such as the NgR. Furthermore, the implanted encapsulated OECs are exposed to additional influencing factors from the CNS.

To confirm our RT-PCR findings, we performed immunocytochemical analysis for Nogo-A and NgR in cultured OECs from flasks. Unlike Nogo-A, the NgR protein could not be detected in cultured OECs. However, the Nogo-A protein was detected only after the cells had been permeabilised, suggesting that OEC Nogo-A is intracellular and is not expressed on the cell surface as in OLGs. The different cellular location of the protein in OECs compared with OLGs may be indicative of an unknown intracellular role. Many studies of Nogo function in inhibiting axonal regeneration have focused on the inhibitory activity of Nogo-A expressed by oligodendrocytes and how it can be neutralized by application of the antibody IN-1 [46, 47]. However, the widespread expression pattern throughout the nervous system and non-neuronal tissues may suggest that Nogo has functional roles unrelated to that already identified. Our finding that the Nogo-A protein is present in the cytoplasmic compartment of OECs, a cell type not normally associated with axonal inhibition, lends further support to this possibility.

In a recent report, immunogold electron microscopy was used to determine the intraneuronal location of the Nogo-A protein [48]. Nogo-A was localized in chromatins within the nucleus and the authors hypothesized that this location suggested a role for the protein in regulation of gene expression. Although further investigation will be required to establish whether Nogo-A is involved in gene expression, it does provide a new perspective on the possible functions of intracellular Nogo.

One consideration is that Nogo expression in OECs could be a function of the ability of OECs to assume a myelinat-

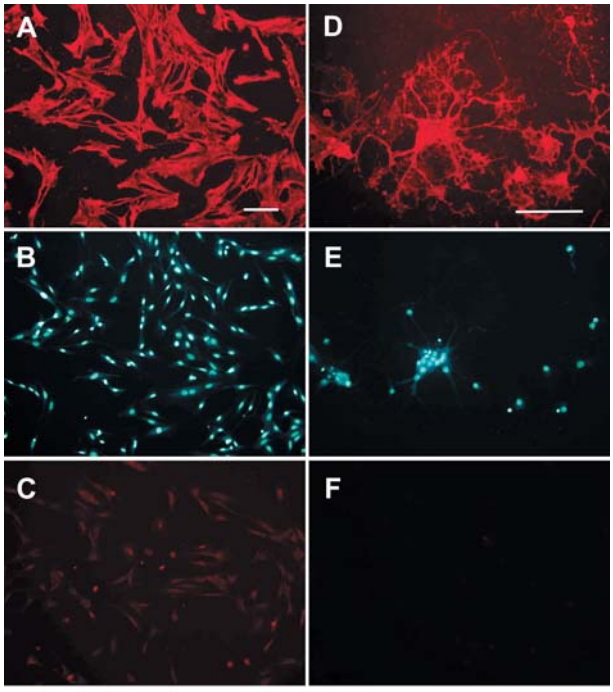

Figure 5. Nogo-A expression in cultured OECs and OLGs. OEC Nogo-A expression was detected intracellularly (*A*). In contrast ,OLG Nogo-A was expressed both on the outer cell membrane and intracellularly (*D*). Both cell types were counterstained with nuclear yellow (*B*, *E*). Controls where the primary antibody was omitted produced negative staining of OECs (*C*) and OLGs (*F*). Scale bar, 100 µm.

ing phenotype under specific culture conditions. Despite the fact that OECs are normally non-myelinating, they have been shown in vitro to myelinate dorsal root ganglion neurites [49]. There have also been several reports suggesting that OECs have the ability to myelinate demyelinated CNS axons in vivo [50–52]. Expression of myelin-associated molecules such as Po [53], myelin basic protein (MBP) [15] and the non-compact myelin protein 2′,3′-cyclic nucleotide 3′-phosphodiesterase [54] in OECs also provides evidence of a myelinating phenotype. However, there have been conflicting reports about the presence or absence of MBP in cultured OECs [55–57].

Recently, the $p75^{NTR}$ receptor was shown to be a co-receptor of NgR in negative signaling by MAG [58]. Given that OECs express both the NgR and $p75^{NTR}$, determining whether these receptors interact in OECs and, if so, investigating the possible function of NgR/$p75^{NTR}$ co-expression in these cells would be useful. However, whether OECs express the NgR protein which was not detectable by immunocytochemical analysis in this study has yet to be established.

We have provided the first quantitative data regarding Nrg-1 and Nogo expression levels in OECs compared with other cell types. Our study also documents the response of cultured OECs at a gene expression level to the injured spinal cord environment. OECs change their expression profile in response to transplantation, suggesting that their phenotype is affected by the lesion environment and therefore that the phenotype of OECs in spinal cord lesions cannot necessarily be inferred from their properties in culture.

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